Interaction between dual specificity phosphatases and JNK scaffolds

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Abstract

The c-Jun N-terminal kinase (JNK) group of mitogen-activated protein kinases (MAPKs) are activated by signals including environmental stresses, growth factors and hormones. In some pathways, scaffold proteins bind JNK and upstream kinases in order to activate subsets of JNK and localise them to specific subcellular sites. For example, the JNK-interacting protein (JIP) scaffold binds JNK, MKK7 and MLKs. The G protein coupled receptor (GPCR) adaptor protein β-arrestin 2 has also recently been identified as a JNK scaffold, binding JNK3, ASK1 and indirectly MKK4. The work presented here shows that JNK specific dual specificity phosphatases MKP-7 and M3/6 bind to JIP-1 and -2 and that MKP-7 can also bind β-arrestin 2. In both cases the phosphatases bind to the scaffolds independently of JNK, using the same region within their extended C terminal domains. MKP-7 can specifically dephosphorylate the β-arrestin 2 bound subset of JNK3 either activated by ASK1 or in response to activation of the GPCR, angiotensin type 1a receptor (AT1aR). MKP-7 transiently dissociates from β-arrestin 2 following AT1aR activation and over expression of ASK1. These results indicate that JIP-1 and β-arrestin 2 modulate JNK signalling by binding JNK-specific kinases and phosphatases. The dynamic interaction between MKP-7 and β-arrestin 2 suggests a possible mechanism by which a positive signal can be passed through a scaffold which binds both activating and inhibitory components.

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Abbreviations

7MSR	seven membrane spanning receptor
AKAPS	a-kinase anchoring proteins
Akt	protein kinase B
Ang II	angiotensin II
AP-1	activator protein-1
AP-2	activator protein-2
Apaf-1	apoptotic-protease activating factor-1
APC	antigen presenting cell
APOER2	apolipoprotein E receptor 2
APP	amyloid precursor protein
ARF-6	ADP-ribosylation factor-6
ARNO	ARF nucleotide-binding-site opener
ASK1	apoptosis stimulating kinase 1
Asp	aspartic acid
AT1aR	angiotensin type 1a receptor
ATF-2	activating transcription factor-2
ATP	adenosine triphosphate
ATPase	adenosine triphosphatase
Bad	Bcl-xL/Bcl-2 Associated Death Promoter
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2 associated X protein
Bcl2	B cell lymphoma
BMK1	big mitogen activated protein kinase
BMP	bone morphogenic protein
bZIP	basic region/leucine zipper
C terminus	carboxy-terminus
CBP	CREB binding protein
CD	common docking
CH2	cdc25 homology
CNS	central nervous system
COOH	carboxy-terminus
CREB	cAMP-response element-binding protein
CRIB	cdc42 rac interacting binding
DCX	doublecortin
DJNK	drosophila JNK
DLK	dual leucine zipper bearing kinase
DMEM	Dulbecco's modified Eagle's medium
DMKP-3	drosophila MKP-3
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate mix
ds	double stranded
DSP	dual specificity phosphatase
DVD	conserved docking site

EDTA	ethylene-diamine-tetra-acetic acid
EGF	epidermal growth factor
EGR-1	early growth response TF
ER	endoplasmic reticulum
ERK	extracellular signal-regulated kinase
ES	embryonic stem
EST	expressed sequence tags
ETaR	endothelin-A receptor
FAK	focal adhesion kinase
FCS	fetal calf serum
FGF	fibroblast growth factor
FHF	fibroblast growth factor homologous protein family
fig.	figure
FL	full length
GADD45β	growth arrest and DNA damage-inducible
GFP	green fluorescent protein
Gly	glycine
GPCR	G-protein coupled receptor
GRK	GPCR kinase
GST	glutathione S-transferase
GTP	guanosine triphosphate
GTPase	guanosine triphosphatase
Grb2	growth-factor-receptor-bound-protein 2
h	hour
HA	haemagglutinin
HBSS	Hanks
Hpk1	haematopoietic progenitor kinase 1
HPLC	high-pressure liquid chromatography
HRPO	horse radish peroxidase
IFN-γ	interferon-gamma
Ig	immunoglobulin
IKK	IκB kinase
IL	interleukin
IL-1R	interleukin-1 receptor
IRS-1	insulin receptor substrate-1
JIP	JNK interacting protein
JNK	c-Jun NH2 terminal kinase
JSAP1	JNK/SAPK associated protein 1
JSP-1	JNK stimulatory phosphatase-1
KAP	kinesin superfamily-associated protein
kDa	kilodalton
KIF	kinesin superfamily protein
KLC	kinesin light chain
LB	luria-Bertani
LDL	low density lipoprotein
LPS	lipopolysaccharide

LZK	leucine zipper kinase
mAb	monoclonal antibody
MAP2K	MAP kinase kinase
MAP3K	MAP kinase kinase kinase
MAPK	mitogen activated protein kinase
MAPKAPK	MAPK activated protein kinase
MAP	mitogen activated protein
MAPs	microtubule associated proteins
MDM2	mouse double minute clone 2
MEF	murine embryonic fibroblast
MEF2c	myocyte enhancer factor 2c
MEK	MAP kinase/Erk kinase
MEKK	MAP kinase/Erk kinase kinase
mGluR	metabotrophic glutamate receptor
min	minutes
MKK	MAP kinase kinase
MKP	MAP kinase phosphatase
MLK	mixed lineage kinase
MNK	MAPK-interacting kinase
mRNA	messenger RNA
MSG	multiple surface glycoprotein
MSK	mitogen and stress-activated protein kinase
-OR	opioid receptor
NES	nuclear export signal
NFAT	nuclear factor of T cells
NF-κB	nuclear factor κB
NGF	nerve growth factor
NH2	amino
NLS	nuclear localisation signal
NSF	*N*-ethylmaleimide-sensitive fusion protein
PAK	P21 activating kinase
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEST	proline (P), glutamic acid (E), serine (S), threonine rich (T)
PHD	plant Homeobox domain
PKA	protein kinase A
PKC	protein kinase C
PMK-1	pathogenicity MAPK
pMKP	phosphorylated MKP
POSH	plenty of SH3 domains
PP2C	protein phosphatase 2C
Pro	proline
PTB	phosphotyrosine binding
pTHR	phosphorylated threonine
PTP	protein tyrosine phosphatase

pTYR	phosphorylated tyrosine
Ral-GDS	Ral GDP dissociation stimulator
Ras-GRF1	Ras-specific guanine nucleotide exchange factor
RhoGEF	Rho guanine nucleotide exchange factor
RIP	receptor-interacting protein
RNA	ribonucleic acid
RNAi	RNA-interference
ROS	reactive oxygen species
RSK	ribosomal S6 kinase
SAM	sterile α motif
SAP	stress activated protein
SAPK	stress activated protein kinase
SDP1	SCAN-domain-containing protein 1
SDS	sodium dodecyl sulphate
Ser	serine
SH3	src homology region 3
SKRP1	SAPK pathway-regulating phosphatase 1
SlpR	slipper
SLT2	suppressor of lytic phenotype 2
SV40	simian virus 40
SYD	sunday driver protein
TAB	TAK1 interacting protein
TAK1	TGF-β activating kinase
TCR	T cell receptor
TF	transcription factor
TGF	transforming growth factor
Thr	threonine
TLR	toll-like receptor
TNF	tumour necrosis factor
TPA	tetradecanoyl phorbol acetate
TPR	tetratricopeptide repeat
TRAF	TNF-associated factor
TRE	TPA response element
Tyr	tyrosine
UV	ultraviolet
VH-1	vaccinia virus phosphatase
VHR	VH-1 related
XIAP	X-linked inhibitor of apoptosis
ZAK	leucine-zipper and sterile-α motif kinase

Chapter 1: Introduction

1.1 Signal transduction

The ability to respond to physical and chemical changes in the environment is an essential property of all cells. These changes range from alterations in nutrients, growth factors, hormones and cytokines to stresses including heat, pH, redox, salt, shearing forces and radiation. Using signal transduction pathways which are activated in answer to these stimuli, cells are able to respond to these changes. These pathways utilise hundreds of proteins which have the ability to interact, modify and activate specific substrates in order to efficiently transmit a signal. Target substrates for these transduction pathways can include transcription factors, the cytoskeleton and other signaling proteins and enzymes. Activation of these substrates can lead to changes in gene expression which can cause up regulation of cellular markers, changes in cell size and shape, cytoskeletal rearrangements and release or retention of extracellular signaling proteins. This ultimately leads to physical cellular responses including migration, proliferation, differentiation, inflammation and apoptosis to name a few.

1.2 Mitogen-activated protein kinases (MAPKs)

A group of proteins that play a particularly important role in response to the environment are the mitogen-activated protein kinases (MAPK) (Schaeffer and Weber, 1999). These MAPKs and the pathways that regulate and respond to them

are evolutionarily conserved through all eukaryotes and play a key role in the regulation of gene expression. The core of the MAPK signaling pathway is a 3-member protein kinase module, consisting of a MAP3K (MAPKK activator), a MAP2K (MAPK activator) and a MAPK (activates substrate outside MAPK module) (fig. 1 - left panel). Upstream pathways which can activate MAP3Ks include the Ras and Rho families of small GTPases. After activation a signal is transmitted through this MAPK module as a sequential phosphorylation cascade culminating in the activation of the MAPK. Originally, it was thought once active the MAPK translocated to the nucleus to activate target substrates which include a variety of transcription factors. However, new data suggest MAPKs can also target cytoplasmic substrates (which will be discussed later). Numerous cellular phosphatases are able to negatively regulate the MAPK and therefore the strength and duration of the output from these cascades. This system allows a cycle between the unphosphorylated and phosphorylated proteins depending on the activity of both the kinases and phosphatases.

The physical and electrostatic forces created by these phosphorylation events markedly alter both the kinase and subsequent substrate protein structures. Protein kinases have a bilobed structure, generally with a small N terminal region and large C terminal region, the catalytic active site region resides in a cleft between these two domains. The crystal structure of the MAPK JNK3 showing the location of these sites is shown in figure 2. Target substrate specificity is dependent on specific domains in the C and N regulatory regions. Both regions contain residues required for binding both substrates and activators. MAPKs are activated by dual

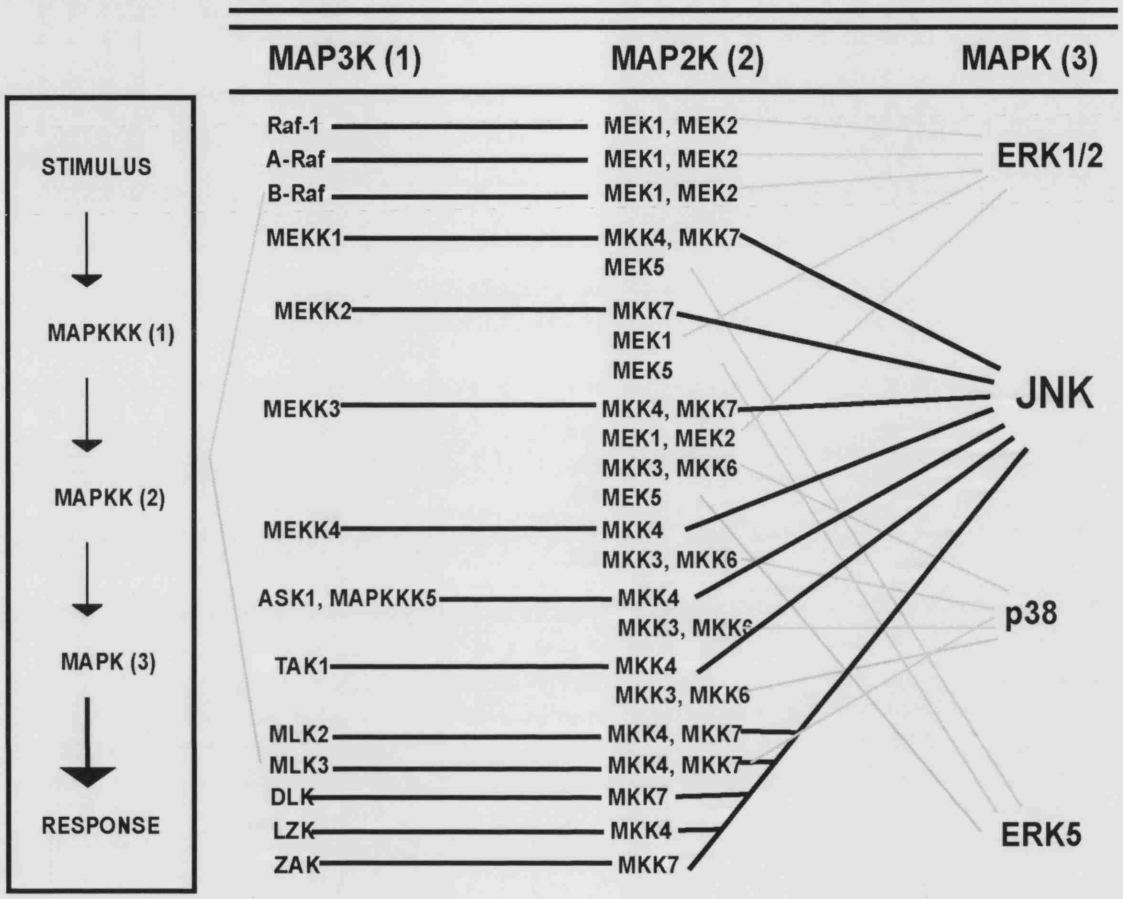

Figure 1 – 1.2 The Mitogen Activated Protein Kinase (MAPK) pathways

Left panel - cartoon representing general signal transmission through different levels of the MAPK cascade.

Right panel – table identifying MAPK targets for each member of the MAP3Ks and MAP2Ks in mammalian cells. Highlighted lines are the proteins and cascades involved in activation of the JNK group of MAPKs.

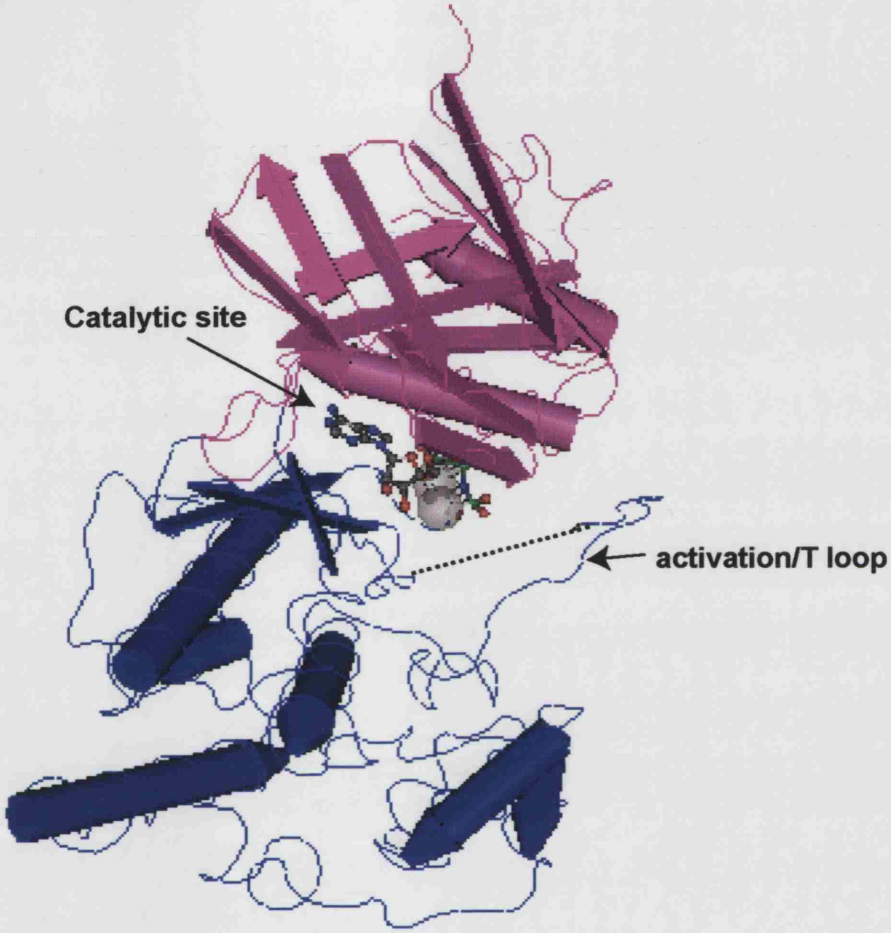

Figure 2 - 1.2 Crystal structure of JNK3

Worm structure representing the non-phosphorylated structure of JNK3 produced by CnD3. Colours are used to represent the N terminal domain (*pink*) and C terminal domain (*blue*). The activation or T loop where the protein is phosphorylated on Tyr and Thr residues by MKK4/7 is shown along with the catalytic site containing AMP-PNP, an ATP analog, (ball-and-stick) and 2 Mg2+ ions in a region between the two domains. Thick Arrows indicate secondary structures present in JNK3. The dotted line indicates a flexible region.

phosphorylation of a tripeptide motif (Thr-X-Tyr) located in the activation or T-loop domain by one or more MAP2K. This specific motif varies both between the distinct groups of MAPKs and within each group; certain isoforms can have these residues differentially phosphorylated by one or more MAP2K. For example, the JNK group of MAPK are phosphorylated by MAP2Ks, MKK4 and MKK7, which preferentially target Tyr and Thr residues, respectively (Lawler et al., 1998). The JNK3 isoform requires dual phosphorylation by both MAP2K for its full activation although phosphorylation solely by MKK7 also leads to significant activation. Contrastingly, phosphorylation only by MKK4 activates JNK3 at a very low level (Lisnock et al., 2000). In addition, MKK7 is preferentially activated by cytokines and MKK4 by environmental stress (Tournier et al., 2001). Therefore, this may be a mechanism under which the JNK cascade can be utilised in response to different stimuli; the cellular response being dependent on the extent of JNK activation.

In order to stabilise the electronegative phosphate group following phosphorylation, the T loop of the MAPK is re-orientated flipping apart and revealing the catalytic domain of the kinase. In this state the kinase is active and can target potential substrates. MAPKs phosphorylate their substrates on phosphoacceptor sites containing Ser/Thr-Pro residues. MAPK substrates also contain docking sites outside of this catalytic domain. These include D-domains, which consist of a cluster of basic residues followed by an LXL motif, and FXF, a short peptide usually located downstream of the phosphoacceptor sites. Some substrates can have either one D-domain or one FXF domain or both. This arrangement adds further specificity to these protein-protein interactions and

allows different subsets of MAPK to target the same substrate. For example, the ternary complex factor Net/SAP-2 has two D-domains; one specifies an interaction with ERK and one with JNK (Ducret et al., 2000).

MAPKs mainly target transcription factor and transcriptional coregulator activity and can do this either in or out of the nucleus. For example, NFAT - nuclear factor of activated T cells, enters the nucleus after dephosphorylation by the protein phosphatase calcineurin. JNK MAPK phosphorylates NFAT4 and NFAT1c to exclude them from the nucleus either through blocking the action of calcineurin or through revealing a nuclear export sequence (NES) (Chow et al., 1997). Other substrates for MAPKs include ribosomal S6 kinase (RSK), mitogen and stress-activated protein kinase (MSK), MAPK-interacting kinase (MNK) and MAPK activated protein kinase (MAPKAPK) which control transcription through the regulation of histones, and transcription factors (Sanchez et al., 1994; Treisman, 1996; Xia et al., 1995).

1.3 MAPK signaling in yeast

In *Saccharomyces cerevisiae* five MAPK pathways have been identified which are activated in response to different environmental signals. These include the mating/pheromone response, filamentation and growth, cell wall remodelling, sporulation and the stress response to high osmolarity (Herskowitz, 1995). Through evolution, *S.cerevisiae* has developed a mechanism where under different stimuli it can utilise a conserved MAPK pathway for two different responses. For example, stimulation with pheromone leads to activation of a specific pathway (STE11, STE7

and FUS3p) resulting in the expression of mating-specific genes. However, members of this MAPK mating response (STE11 and STE7), under alternative stimulation, can also elicit responses targeting the MAPK KSS1 for the invasive growth response. This provides yeast cells with an ability to generate pathway specificity, even when pathway module components have multiple roles (Madhani et al., 1997). Evidence generated in yeast to understand the function of MAPK pathways has allowed the identification of similar systems in mammalian cells. Complementation experiments where mammalian MAPKs replace their yeast equivalents and function correctly identify a clear evolutionary conservation of MAPK function between yeast and mammalian systems (Galcheva-Gargova et al., 1994). However, the need to respond to more stimuli in mammalian cells has led to the development of more complex MAPK pathways. In mammalian cells, JNK and p38, are responsible for the stress response, whereas in fission yeast Hog1 and Slt2 are the stress-activated MAPK (Brewster et al., 1993). Similarly, there is also an increase in the number of MAP3K, MAP2K and MAPK substrates involved in mammalian MAPK signaling.

MAPK signaling modules exist ubiquitously in all mammalian cells. However, the core proteins are differentially utilised by different cell types for different responses. Examples discussed below clearly identify the mammalian stress-activated MAPK JNK as being able to induce opposing responses in both apoptosis and survival. In some cases scaffold proteins are utilised by MAPK pathways to localise, organise and control the signaling components required to elicit the desired response. By expressing certain combinations of MAPK isoforms, a

cell is primed to convert a particular stimulus into a specific transcriptional response. In this way distinct cell types can respond in alternative ways and magnitudes to the same stimulus. Negative MAPK regulators can also be differentially expressed to control the duration of MAPK activation and therefore the resulting response. Throughout this introduction examples of how MAPK modules are activated and the subsequent physiological response are discussed. It is important to take into account this is potentially only true in the cell type examined.

1.4 Mammalian MAPKs

So far three major groups of mammalian MAP kinases have been identified. These are the extracellular signal-regulated kinase (ERK1/2), the c-Jun NH2-terminal/Stress Activated Protein Kinase (JNK/SAPK) and the p38 group of MAPKs. Each MAPK, as shown in figure 1, has a group of activating MAPKKs, which in turn are targeted for phosphorylation by MAPKKKs (Chang and Karin, 2001). As well as these specific upstream activators, other important mechanisms have been described in both yeast and mammals which provide MAPK specificity. These include docking mechanisms and scaffold proteins.

However, as expected, transmission through one group of MAPK is not always unique to one stimulus. For example, the ERK group of MAPKs are known to be activated in response to growth factors, phorbol esters and hormones, whereas the JNK and p38 MAPKs are activated by stresses including osmotic & redox shock and changes in pH (Kyriakis and Avruch, 2001). In some cells the opposite

situations occur, heat shock and UV radiation can activate ERK (Huang et al., 1999) and epidermal growth factor (EGF) can activate both JNK and p38. Another example of the complexity of MAPK signaling is shown through the identification of the Ras-dependent activation of both ERK and JNK MAPKs through different MAP3K (Minden et al., 1994). MAPK signaling can also work through feedback mechanisms. For example, sustained JNK activation through MLK3 by TNFα leads to the uncoupling of the MEK-to-ERK pathway through activation of c-Jun (Shen et al., 2003). This results in the inhibition of ERK activation in response to EGF and suggests MAPKs can mediate responses both through negative feedback of opposing pathways as well as activation of their own specific substrates. However, in cells deficient of JNK, there is no difference in the activation of ERK or p38 in response to TNFα in spite of complete inhibition of JNK activation suggesting any link between the MAPK groups in response to TNFα is not crucial (Ventura et al., 2003).

1.5 The c-Jun NH2 terminal Kinase (JNK) group of MAPKs

1.5.1 Stimuli

The JNK group of MAPK kinases are important for many cellular responses including apoptosis, growth, survival, differentiation, embryonic development and the immune response (Davis, 2000; Dong et al., 1998). In particular, JNK is potently activated in response to stress signals, such as UV radiation, heat, osmotic and ribotoxic shock, ischemic events, chemotherapeutic agents, other protein and DNA

damaging stresses (Adler et al., 1995; Cavigelli et al., 1996; Derijard et al., 1994; Galcheva-Gargova et al., 1994; Hibi et al., 1993; Yoshida et al., 2002), as well as more transiently by proinflammatory cytokines like TNFα, dsDNA, lipopolysaccharide, interleukin-1, small G proteins, ceramide, hormones and growth factors (Cavigelli et al., 1996; Chu et al., 1999; Coso et al., 1995; Guan et al., 1996; Sluss et al., 1994; Westwick et al., 1994).

1.5.2 Substrates

The main downstream transcriptional target of JNK is the transcription factor c-Jun, a member of the AP-1 (activator protein-1) family of transcription factors. JNK was originally identified as being a protein which could phosphorylate and activate c-Jun (Hibi et al., 1993). Other members of this family include Fos, JunB, JunD and ATF2 (Davis, 2000; Ventura et al., 2003). c-Jun is the central component of all AP-1 complexes; and is expressed in many cell types at a low level. Its expression is induced in response to stimuli including growth factors, cytokines and UV radiation. The presence of bZIP domains in the AP-1 family allows, after phosphorylation, the formation of homo- and heterodimers between AP-1 members which generate transcription-competent AP-1 complexes. The composition of these complexes determines the target gene spectrum and physiological response (Shaulian and Karin, 2002). In resting cells, JNK is located in both the cytoplasm and nucleus. Upon activation, for example with UV radiation, JNK accumulates in the nucleus. To activate c-Jun, JNK binds to the activation domain within the NH2 terminal region and phosphorylates Ser-63 and -73 (Pulverer et al., 1991). c-Jun also

requires dephosphorylation at specific C terminal residues to enable its DNA-binding activity (Boyle, 1991). The target sites for JNK phosphorylation in other substrate transcription factors correspond to the Ser/Thr-Pro motif within the activation domain of c-Jun. Specific activation of c-Jun by JNK requires additional binding residues collectively termed the δ-domain which lies upstream of the phosphorylation sites (Hibi et al., 1993; Kallunki et al., 1994). Once phosphorylated, c-Jun forms a dimer with other AP-1 family members, including ATF-2. The c-Jun-ATF2 complex recruits CREB binding protein (CBP) and then binds the TPA-response elements (TREs), Jun1 and Jun2 to enhance transcriptional activation.

JNK also phosphorylates other transcription factors including Elk-1. JNK also contributes to gene regulation indirectly by influencing histone acetylase activity via protein kinases (Kawasaki et al., 2000) as well as regulating ubiquitin-mediated degradation of the AP-1 protein (Fuchs et al., 1998). JNK has also been shown to target the tumour suppressor protein p53 for phosphorylation. It is suggested JNK can activate p53 during UV stimulation as way of activating apoptosis (Hu et al., 1997). JNK can also target cytoplasmic substrates including microtubule-associated proteins (MAPs) (Chang et al., 2003), 14-3-3 proteins (Tsuruta et al., 2004) and Bcl2 family members (Yu et al., 2004) and the protein kinase RSK in order to activate glycogen synthase in response to insulin challenge (Moxham et al., 1996)

1.5.3 JNK characterisation

In *Drosophila melanogaster* embryonic development, JNK is required for fusion of epithelial sheets during the process of dorsal closure as well as in the apoptotic

program required for limb patterning (Igaki et al., 2002; Martin, 2002) and apoptosis induced by the TNF superfamily homolog, Eiger (Moreno, 2002). In *Caenorhabditis elegans*, the JNK-homolog plays a role in the co-ordination of body movement, synaptic vesicle transport and in the stress response (Mizuno et al., 2004).

Mammalian JNK is encoded by three genes. The JNK1 and JNK2 genes are expressed ubiquitously whereas JNK3 has a limited expression pattern localised to the CNS, as well as low level expression in both the heart and testes. These genes express 10 different isoforms through alternative splicing (Gupta et al., 1996). The splice variants of all three genes can encode proteins both with and without COOH tail domain extensions thus producing 46 kDa and 55 kDa proteins. The JNK1 and JNK2 genes have further splicing variations involving the kinase domain. This alteration influences the substrate specificity of JNK1 and JNK2 by changing docking domains required by JNK to interact with multiple upstream activators and downstream targets (Tanoue et al., 2000).

Interestingly, the JNK1 and JNK2 proteins have different binding affinities towards their substrate c-Jun. In fact JNK2 is 25 times more inclined to interact with c-Jun than its family member JNK1 (Kallunki et al., 1994). Different tissues express different levels of the JNK isoforms, indicating they may have cell specific functions. Disruptions of the JNK genes in mice have identified a certain level of compensation between the proteins, as well as identifying unique tissue and signaling profiles for each isoform. Mice deficient in JNK1 or 2 are severely immunodeficient due to defects in T-cell function (Constant et al., 2000). These defects are due to a dysfunction in effector T-cells not their proliferation, as

peripheral JNK1/2 -/- T-cells have a higher proliferation rate and produce more IL-2 than wild type cells (Dong et al., 2000). Compound mutations of JNK1 and JNK2 are embryonic lethal indicating JNK plays a critical role in mouse development. MEFs deficient in JNK1 and JNK2 are resistant to UV, proteosome inhibition and genotoxic drug induced apoptosis. JNK is thought to engage the Bax/Bak apoptosis pathway as Bax/Bak knockout MEFs are resistant to ectopic expression of a MKK7-JNK constitutively active fusion protein, whereas wild type MEFs are not (Lei et al., 2002). Mice with a JNK3 gene disruption develop normally but show an increase in resistance to neuronal apoptosis and seizure activity in response to excitotoxins. A considerable reduction in phosphorylation of c-Jun and formation of the AP-1 transcription factor complex was also seen in these JNK3 -/- mice (Yang et al., 1997b). As its absence is neuroprotective, JNK3 could therefore be a therapeutic target for many neuronal injuries including stroke (Kuan et al., 2003), Parkinson's (Hunot et al., 2004) and Alzheimer's disease (Morishima et al., 2001).

1.5.4 The role of JNK

1.5.4.1 Apoptosis

As indicated through the knockout studies described above, one physiological role of JNK is in the regulation of apoptosis (Wada and Penninger, 2004). For example, nerve growth factor (NGF) deprivation in PC12 cells leads to sustained JNK activation and induction of apoptosis. Furthermore, the presence of dominant negative c-Jun or MAP3K MEKK1 protects these cells from apoptosis after NGF

withdrawal (Xia et al., 1995). Biochemical studies used to analyse the function of JNK in apoptosis suggests a relationship between activation of JNK and mitochondrial compromise leading to cell death (Tournier et al., 2000). For example, exposure to UV radiation causes the release of cytochrome C from depolarised mitochondria, which acts together with Apaf-1 to activate caspase-9 which in turn activates caspases 3, 6 and 7. During this type of response JNK was shown to phosphorylate c-Jun but no new transcription was detected (Tournier et al., 2000), suggesting JNK does not mediate the UV radiation response through gene regulation. A few possible JNK targets involved in this process have been identified including the Bcl2 group of apoptotic regulatory proteins (Lei and Davis, 2003; Lei et al., 2002; Maundrell et al., 1997) and the tumour suppressor p53 (Hu et al., 1997). Alternatively, it has been suggested c-Jun can regulate the re-entry into the cell cycle from p53-induced growth arrest (Shaulian, 2000). The essential downstream targets of JNK in apoptosis have yet to be clearly identified. Therefore, JNK's precise role in apoptosis remains controversial as it appears to be dependent on species, cell type and nature of apoptosis stimulus. Late passage MKK7 -/- mutant cells are resistant to apoptosis similar to JNK knockouts, however earlier passages rapidly undergo apoptosis similar to wild type cells (Wada and Penninger, 2004). Some studies report that the same molecule in the same cell line can give an opposing result. For example, c-Jun deficient MEFs show more spontaneous cell death compared to their wild type counterparts indicating a role for c-Jun in survival. However, these MEFs are also resistant to UV induced cell death suggesting a role in apoptosis (Shaulian, 2000).

JNK activation changes during tumour growth, implying proteins that regulate JNK could act as tumour suppressor proteins. The oncogenic potential of this pathway was confirmed when the JNK activator MKK4 was identified as a tumour suppressor and metastasis suppressor candidate in both breast, prostate and pancreatic cancers (Cazillis et al., 2004; Wang et al., 2004; Yoshida et al., 1999). For example, in ovarian cancer MKK4 expression is significantly decreased when compared with normal cells (Yamada et al., 2002).

1.5.4.2 Survival

Therefore JNK can also play a role in cell survival; the exact nature of this however is unclear. It seems cells undergo apoptosis after prolonged JNK activation (UV, ribotoxic shock), whereas transient JNK phosphorylation may be responsible for the role of JNK in survival, proliferation and differentiation (cytokines, growth factors). The precise mechanism of how JNK is involved in survival is not clear. However, JNK has been shown to play a role in IL-3-mediated cell survival through inactivation of apoptosis inducer BAD (Yu et al., 2004). Whether chronic or acute JNK activation occurs, JNK can be regulated through feedback systems which could also play a part in JNKs role in survival including NF-κB. For example, in wild type MEFs, TNFα induces activation of NFκB and a transient phosphorylation of JNK. By removing the RelA subunit of NFκB from these cells, TNFα can induce the sustained activation of JNK which results in apoptosis (Reuther-Madrid et al., 2002). Ectopic expression of either XIAP (X chromosome-linked Inhibitor of Apoptosis) or GADD45β (proteins induced by TNFα in a NFκB dependent manner) inhibits the

prolonged activation of JNK seen in TNFα stimulated RelA -/- MEFs (De Smaele et al., 2001). Furthermore, GADD45β has been shown to inhibit JNK activation by TNFα by specifically binding the MAP2K MKK7 and competing for the surrounding ATP, therefore blocking the TNFα signal transmission to JNK (Papa et al., 2004).

The JNK substrate c-Jun was originally cloned as a proto-oncogene (Bohmann et al., 1987) as it is highly expressed in some tumours where it induces proliferation (Mathas et al., 2002). c-Jun has been shown to abrogate the expression of tumour suppressor protein p53 in MEFs (Schreiber et al., 1999). In addition, JNK can also activate the tumour suppressor protein, p53 leading to apoptosis. Therefore loss of JNK could result in increase in proliferation and survival. These studies indicate the complexity of JNKs physiological role and its regulation and also acutely shows how difficult it is to assign one general function to JNK which is correct for all cell types and stimuli.

The remainder of this introduction will discuss proteins which act both positively and negatively towards JNK in order to regulate its activation. These include the MEKKs, MLKs, MKKs, scaffold proteins and dual specificity phosphatases.

1.6 The MAP2K members that phosphorylate JNK

MAP2Ks that phosphorylate MAPKs include MKK3/6/4 which target p38s, MEK1/2 which target the ERKs and the most recent discovery MEK5 which targets ERK5. JNK is directly activated through the dual phosphorylation of residues threonine (Thr) and tyrosine (Tyr) of a TPY motif located in the activation loop by upstream MAP2Ks, MKK4 and MKK7 (Lin et al., 1995; Tournier et al., 1997)(fig. 1). Under environmental stress JNK is activated by MKK4 whereas MKK7 activates JNK in response to cytokine stress including TNFα and IL-1 (Moriguchi et al., 1997; Tournier et al., 2001). Although MKK4 and MKK7 activate JNK as dual specificity kinases, they preferentially phosphorylate the Tyr and Thr residues, respectively (Lawler et al., 1998). Potentially, the extent of Thr and Tyr phosphorylation is dependent on a sequential phosphorylation mechanism which could control the activated state of JNK (Kishimoto et al., 2003). It is therefore possible, as described earlier, that MKK4 and MKK7 cooperate under particular JNK stimuli to give the appropriate level of response or activate JNK differentially to target JNK to one of its diverse roles.

The upstream activators of MKK4 and MKK7 are shown in figure 1 and described below. Activation of MAP2Ks occurs via phosphorylation at two sites in the T-loop by the upstream MAP3K. As ser/thr kinases, the MKKs exhibit similar structural and functional features to those of the MAPKs. There are three MKK4 protein kinase isoforms with unique N terminal domains, and six MKK7 isoforms with different C and N terminal domains. These variations exist due to the alternative splicing of these genes as well as the use of different gene promoter

sequences (Tournier et al., 1999). The general structures of MAP2Ks are shown in figure 3. They all retain the ability to activate JNK but vary in their basal activity and inducibility. JNK can interact specifically with MKK4 through a conserved docking (CD) domain consisting of a cluster of acidic residues at the C terminus of JNK, which interacts with a region of basic residues in the N terminal of MKK4 (Tanoue et al., 2000). This is retained through many MAP2K-MAPK interactions and more recently additional sequences have been shown to be required for specificity (Tanoue et al., 2001a). A domain within the C terminal region of MKK4 and -7 termed DVD (conserved docking site), has been identified as a site required for binding to and being activated by MAP3K (Takekawa et al., 2005). The cellular localisation of MKK4 and MKK7 is cytoplasmic but under some stresses they have been shown to accumulate in the nucleus (Tournier et al., 1999). This suggests that JNK can be activated both in the nucleus and cytoplasm. These MAP2K have been shown to play a role in scaffold activation of JNK. MKK7 but not MKK4 has been shown to interact with the JIP (JNK interacting protein) set of proteins (Whitmarsh et al., 1998), whereas MKK4 complexes with the ASK1-JNK3 on the β-arrestin 2 scaffold protein (McDonald et al., 2000).

Gene disruption in mice of either MKK4 or MKK7 demonstrates an essential function in embryonic development (Yang et al., 1997a). Loss of viability in MKK4 null mice is thought to be caused by massive apoptosis in hepatocytes. This results in the failure of liver formation during early development (Nishina et al., 1999). There are conflicting reports about the role of MKK4 in the immune system. Some evidence suggests MKK4 null mice are defective in T cell IL-2 secretion and

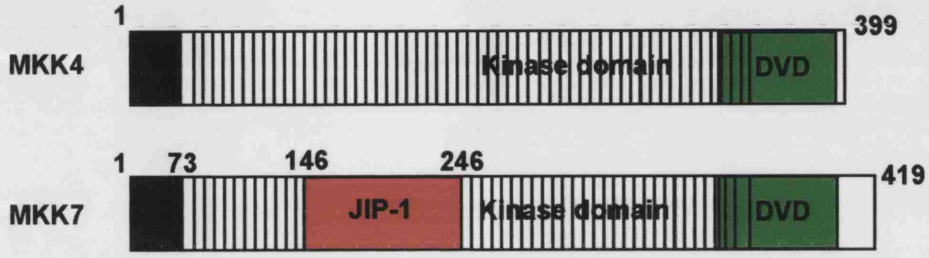

Figure 3 – 1.6 The JNK MAP2K, MKK4 and MKK7

Cartoon showing general sites present in MKK4 and MKK7. *Numbers* refer to the amino acid position. Also shown are the proposed regions required for binding JNK and DLK (*black*), JIP-1 (*red*), MAP3K – including MEKK1 (*green)* and kinase domains (*striped*). *DVD,* conserved docking domain. It is important to take in to account both these proteins have numerous isoforms as described in the text and that this illustration only shows a general structure.

proliferation (Nishina et al., 1997). However, this was not confirmed in subsequent studies which showed T cells develop normally and exhibit normal JNK activation (Swat et al., 1998). This might, however, suggest MKK7 may compensate for the loss of MKK4 in these cells and vice versa. However, this is not seen in mast cells lacking in MKK7 as they have no detectable JNK activity even though MKK4 expression is upregulated (Sasaki et al., 2001). Compound mutations of both MKK4 and MKK7 will be essential to fully understand the relationship between these proteins and their roles in JNK activation.

Recently, the MKK7-JNK-c-Jun pathway has been identified as a key link between cellular stress, development and senescence (Wada et al., 2004); some data also suggest MKK4 may have a similar role. As described above, MKK4, as a regulator of JNK, has been identified as a tumour suppressor in many cancers. However, MKK7 and MKK4 are activated by numerous upstream kinases and therefore each physiological role these proteins have, may be dependent on which upstream kinase activates them. These interactions will be discussed below.

1.7 The MEKK group of MAP3K

The MAP3K MEK kinase group (including MEKK1), was one of the first MAPKKK protein families to be identified (Xu et al., 1996). The most diverse group of MAP3Ks, MEKKs have an N terminal non-catalytic domain followed by a common conserved catalytic domain at the C terminus end (fig. 4), which is highly homologous to the *S. cerevisiae* MAP3K, STE11. Most of the MEKK family members have not been sufficiently characterised to fully identify functional elements within

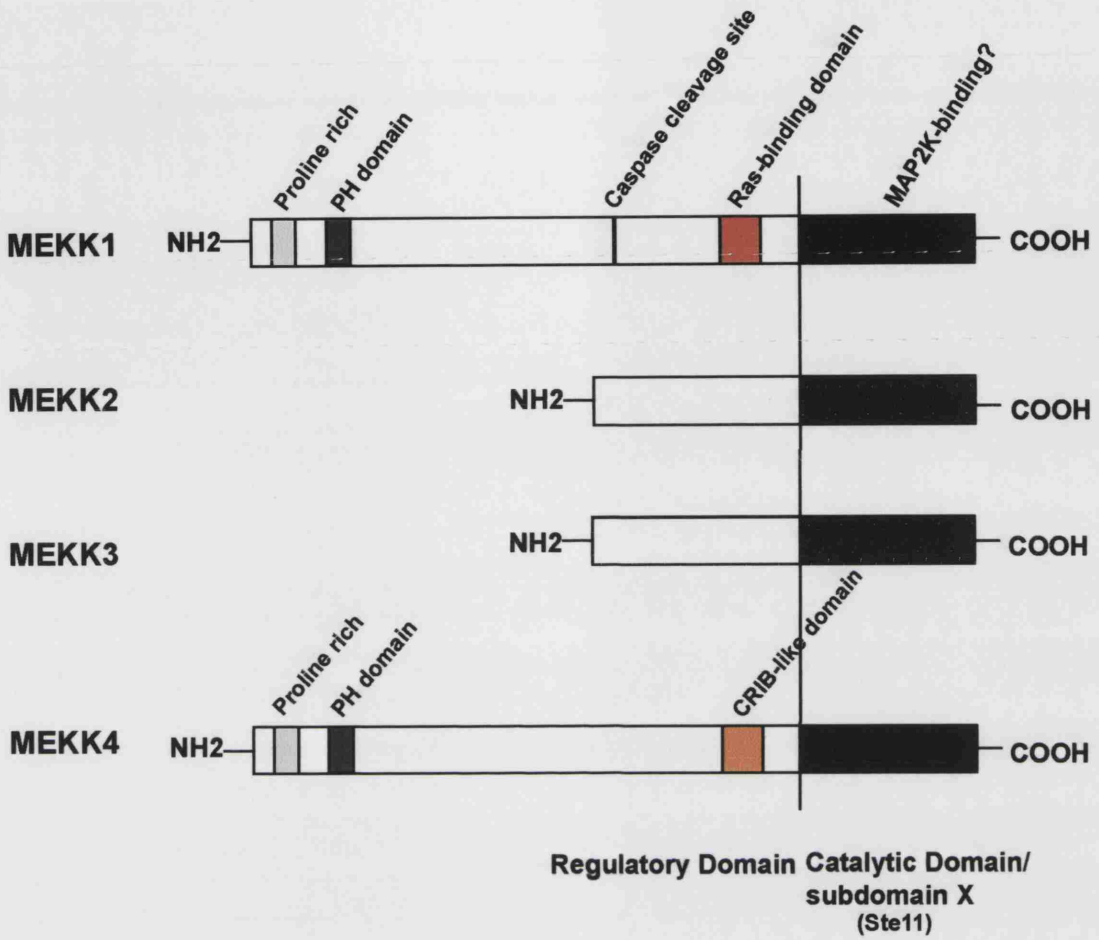

Figure 4 – 1.7 MEKK proteins

Cartoon showing four members of the MEKK family. The catalytic domain exists in the C terminal region and is highly homologous to Ste11. Numerous sites are present in the N terminal regions that regulate binding to substrates including GADD45-like proteins in MEKK4. The proline rich domains are functionally significant as they control interactions with proteins containing SH3 domains. *PH* – indicates Plant Homeobox domain.

their N terminal domains. Although it has been shown these regions do contain both binding and regulatory domains. This group of MAP3K are varying in their degree of promiscuity towards downstream substrates, MAP2Ks. Furthermore, the MEKKs also have different subcellular localisations, for example MEKK1 localises to the plasma membrane, MEKK2 and -3 to the cytoplasm and MEKK4 seems to have a golgi-like/perinuclear distribution (Fanger et al., 1997).

Initially, thought to be a strong activator of the ERK MAPK cascade, MEKK1s catalytic domain has since been shown to preferentially activate JNK (Minden et al., 1995; Minden et al., 1994), via the MAP2K, MKK4 (Derijard et al., 1995; Yang et al., 1997a) and to a lesser extent MKK7. The catalytic site of MEKK1 has been shown to contain residues that are conserved in other MAP3K including P21 activated kinase (PAK1) and protein kinase Aα (PKAα). This site, known as subdomain X, is critical for MEKK1 interaction and activation of MKK4 (Tu et al., 2003). Interestingly MEKK1 binds directly to both JNK and MKK4, suggesting it has a role as a scaffold protein forming a three member MAPK signaling module (Xu and Cobb, 1997). MEKK1 is a 196-kDa serine-threonine kinase that contains a long N terminal domain containing many protein interaction sites and a catalytic C terminal domain. As above, the interaction domains of the MEKKs reside in the regulatory N terminal domain and have been relatively uncharacterised but include binding elements for upstream signaling and adaptor proteins Grb2 (Pomerance et al., 1998), Rac/Cdc42 (Fanger et al., 1997), PAK1 (Gallagher et al., 2002), other PAK1-like proteins (Su et al., 1997), Ras (Lange-Carter et al., 1993), Rho GTPases (Gallagher et al., 2004) and 14-3-3 proteins (Fanger et al., 1998). MEKK1 contains a Plant

Homeobox Domain (PHD) which has been shown to contain E3 ligase activity which targets ERK1/2 (Lu et al., 2002). MEKK1 itself is a substrate for caspase-3. Upon cleavage at Asp 874 the 91kDa COOH domain is released causing further activation of caspase-3 and subsequent induction of apoptosis (Widmann et al., 1998). Furthermore, MEKK1 is also an important regulator of JNK under TNFα and IL-1 stimulation via TRAF2 & 6 respectively (Baud et al., 1999), as well as other proinflammatory cytokines and growth factors (Fanger et al., 1997; Xia et al., 2000). Both the apoptosis stimulating kinase (ASK1) (Nishitoh et al., 1998) and TGF-β activating kinase (TAK1) (Takaesu et al., 2000) (both members of the MEKK family) have also been identified as associating with these same TRAF and other adaptor proteins, respectively. Therefore these MAP3K have the ability to activate JNK using the same signaling network but possibly under alternative stimulation or for an alternative response.

Furthermore, MEKK1 has also been identified as a regulator of the JNK response in survival against apoptosis induced by reperfusion after an ischemic event. The reperfusion event produces reactive oxygen species (ROS). ROS damage is caused by its ability to oxidise proteins as well as stimulate the production of TNFα, which is capable of inducing apoptosis in this environment. In the presence of ROS, MEKK1 -/- embryonic stem cell derived cardiac myocytes show a marked reduction in JNK activation. This leads to the suppression of TNFα expression induced by p38 thus giving MEKK1, and therefore JNK, a role in the survival of cardiac myocytes when damaged by ROS (Minamino et al., 1999). It seems MEKK1 also protects cells from stresses which alter cellular shape (Yujiri et al., 1999) as well

as cold stress but not heat shock, UV radiation or anisomycin (Yujiri et al., 1998). MEKK1 can also target non-MAPK pathways. In particular, MEKK1 is a downstream target of Bcr-Abl and is responsible for mediating cell survival in Bcr-Abl transformed cells via the NF-κB pathway (Nawata et al., 2003). These roles of MEKK1 in stress induced signaling i.e. its ability to induce apoptosis as a substrate for caspase-3 and alternatively its role in survival against oxidative stress, further suggest the complexity and the multiple cross over and roles of proteins within this group of signaling molecules.

MEKK2 and -3 are more promiscuous MEKKs, activating JNKs, p38s and ERKs, as well as ERK5. Smaller proteins, 70 and 71 kDa respectively, these protein kinases share ~90% homology between their catalytic domains (Blank et al., 1996). The mechanism of MEKK activation and regulation in general is not clear. However, the ubiquitin Ligase, Smurf1, regulates osteoblast activity through targeting MEKK2 for ubiquitination and therefore degradation (Yamashita et al., 2005). In another instance, the catalytic domain of MEKK2 allows the formation of inactive MEKK2 homo-dimers. Phosphorylated or activated MEKK2s are less likely to form these dimers suggesting this may be a mechanism by which MEKK2, and therefore its substrates, are regulated (Cheng et al., 2005). MEKK2, preferentially activates JNK via MKK7 (Cheng et al., 2000) but also ERK1/2 via MEK1 and ERK-5 via MEK5. Whereas MEKK3 can activate nearly every MAPK pathway via different MAP2K (Chao et al., 1999; Deacon and Blank, 1997; Deacon and Blank, 1999). As discussed earlier, JNK functions within the immune response particularly in T-cell function and differentiation. In T cells, MEKK2 is activated by translocation to the

TCR-APC interface, and is required for activation of both ERK and p38 cascades (Schaefer et al., 1999). Interestingly, both endogenous MEKK1 and over expressed MEKK3 are unable to translocate to the plasma membrane in this system. This demonstrates that the N terminal region, not the catalytic domain of MEKK2, which is 94% homologous to MEKK3, contains undetermined elements which target MEKK2 for this function. However, T-cells from mice deficient of MEKK2 under anti-CD3 Mab stimulation proliferate more and produce more IL-2 and IFN-γ than wild type cells (Su et al., 2001). These cells do not inhibit TCR/CD3 induced JNK activation but do in fact enhance JNK activity by 3-4 fold suggesting that MEKK2 could control TCR signaling through negative regulation of JNK and not ERK or p38 (Guo et al., 2002). In MEKK2 -/- ES-derived mast cells, activation by IgE and c-kit fail to elicit the appropriate cytokine response and receptor-mediated JNK activation (Garrington et al., 2000). In the same study, JNK activation is undisrupted in response to UV radiation stress, suggesting MEKK2 is a crucial JNK activator under receptor stimulation and yet does not play an important part in JNK activation by UV radiation. A further study looking at activation of mast cells by the cross linking of FCεR1, identifies MKK7, not MKK4, as the downstream kinase of MEKK2 in receptor-mediated JNK activation and interestingly MEK5-ERK5 as the downstream pathway of MEKK2 in the regulation of TNFα gene expression (Chayama et al., 2001).

MEKK3 can activate all groups of MAPK. In particular, MEKK3 has been shown to regulate JNK and IKK-NF-κB, but not ERK, signaling through IL-1 and LPS mediated activation of IL-1R and TLR4 (Huang et al., 2004). MEKK3 plays a

critical role in embryonic blood vessel development through activation of Mef2c; Mef2c is a transcription factor crucial for early cardiovascular development through the p38 MAPK cascade (Yang et al., 2000). MEKK3 has also been described as a key regulator of TNF-induced NF-κB activation via interaction with receptor-interacting protein (RIP) and subsequent phosphorylation of the I-κB Kinase (IKK) (Yang et al., 2001). Subsequent studies have confirmed these results identifying specific interactions made by MEKK3 and not MEKK2 with I-κB which lead to regulation of NF-κB (Schmidt et al., 2003). Furthermore, this regulation of NF-κB may lead to the resistance of apoptosis in both breast and ovarian cancers (Samanta et al., 2004).

Two splice variants of MEKK4, α and β, are widely expressed in mouse cells. An approximately 180 kDa protein, its kinase domain is 80% homologous to MEKK1 but unlike MEKK1 the NH2 domain contains identified protein interaction domains, including a CRIB-like domain (Gerwins et al., 1997) and interaction sites for three related GADD45-like proteins (Takekawa and Saito, 1998). The relationship between GADD45β/GADD45γ and MEKK4 was confirmed recently, suggesting it regulates IFNγ production via p38 in T cells (Chi et al., 2004). MEKK4 preferentially activates JNK and then p38, but not the ERKs unlike the other MEKK family members. It activates JNK following interaction with Cdc42/Rac via its CRIB-like domain, and subsequent JNK activation is blocked by the addition of a kinase dead MKK4, suggesting MEKK4 activates JNK via a sequential phosphorylation through MKK4 (Gerwins et al., 1997). MEKK4s cellular function is even less defined than other members but it has been linked to embryonic differentiation in response to retinoic acid (Kanungo et al., 2000) as well as response

to environmental stresses including UV and γ-irradiation (Takekawa and Saito, 1998). More recently, MEKK4 was shown to play a significant role in neural tube development through activation of JNK specific MAP2K, MKK4 (Chi et al., 2005).

Studies attempting to characterise the MEKKs have concentrated on analysing the kinase domains of these proteins in order to identify downstream targets and binding proteins. However, as shown above the N terminal domains are significantly different suggesting that they contain sites required for the maintenance of signal specificity (Bonvin et al., 2002). Although the MEKKs do regulate JNK activity it is clear this group of proteins are involved in many other signaling pathways and so understanding their functions remains crucial.

TAK1 and ASK1 are other members of the MEKK family. TAK1, a small 60-kDa protein, activates both JNK and p38, via MKK4, -3 and -6 (fig. 1) (Moriguchi et al., 1996; Wang et al., 1997). This MAP3K is also alternatively spliced resulting in 4 isoforms with different C terminal regions. TAK1 is activated under stimulation by transforming growth factor-beta (TGF-β), Toll-like receptor (TLR) ligands and bone morphogenic protein (BMP) (Yamaguchi et al., 1995). It is also involved in activation of NF-κB in response to IL-1 (Ninomiya-Tsuji et al., 1999) and LPS (Irie et al., 2000). TAK1 interacting proteins (TAB1 and -2) also implicate TAK1 in TGF-β signaling (Shibuya et al., 1996; Takaesu et al., 2000) as well as in development (Shibuya et al., 1998) and stress signaling (Wang et al., 2001). ASK1 is a ubiquitously expressed MAPKKK that activates JNK by directly activating MKK4 (Ichijo et al., 1997; Wang et al., 1996). It has been implicated in apoptosis in response to oxidative, endoplasmic reticulum (ER) and genotoxic stress as well as by TNFα and

Fas ligand (Chen et al., 1999; Nishitoh et al., 2002; Nishitoh et al., 1998; Tobiume et al., 2001). Other biological roles include decisions of cell fate such as differentiation and survival (Sayama et al., 2001; Takeda et al., 2000). A 150 kDa protein, ASK1 includes a central kinase domain flanked by an NH2 extension which contains binding sites for TRAFs, the Fas-associated adaptor protein Daxx (Chang et al., 1998) and the redox sensing enzyme thioredoxin (Saitoh et al., 1998). ASK1 is regulated by its ability to bind the reduced form of thioredoxin via this domain; an ASK1 mutant containing no NH2 domain is a constitutively active kinase. It is thought that activation of ASK1 by oxidative stress and TNF is the result of a change in the redox state of thioredoxin caused by ROS, thus releasing ASK1 and converting a redox signal into a MAPK signaling pathway (Gotoh and Cooper, 1998; Liu et al., 2000a).

1.8 The MLK group of MAP3K

The Mixed Lineage Kinase (MLK) MAP3Ks are the best studied group of MAP3Ks and were named as such because of they contain a mismatch of classical kinase domains. The primary sequences of subdomains I-VII of the MLKs resemble serine/threonine kinases whereas the remaining 4 domains resemble tyrosine kinases (Dorow et al., 1993). Eight MLK members have been identified and subsequently due to their domain arrangements and sequence similarities they have been further classified into 3 subgroups: MLKs (MLK1-4), DLKs (DLK & LZK) and ZAKs (ZAKα & -β). These are shown in figure 5 (Gallo and Johnson, 2002).

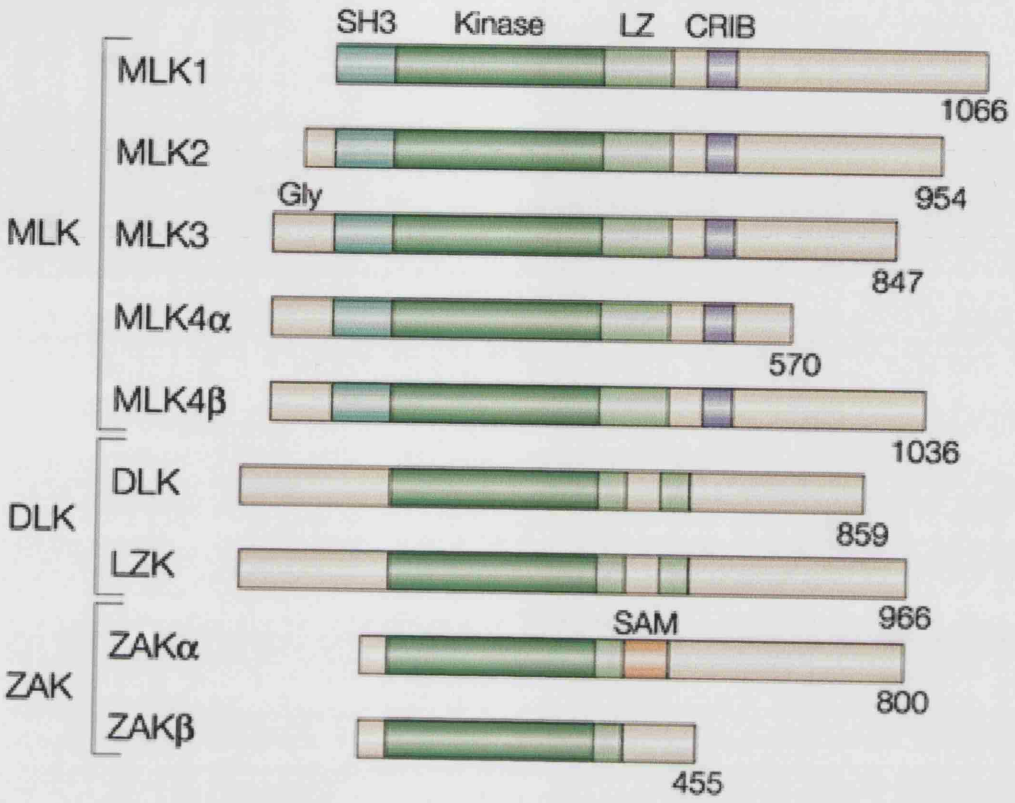

Figure 5 – 1.8 The MLK proteins

Cartoon showing members of the MLK family. The names are indicated on the left hand side. *Numbers* refer to amino acid position. Domains shown include Src-homology (SH3); kinase; leucine zipper (LZ); Cdc42/Rac-interactive binding (CRIB); sterile-α motif (SAM).

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So far *Drosophila* is the only lower eukaryote identified as utilising a protein similar to members of the MLK family. Known as Slipper (Slpr) this MLK-like protein has 50% amino acid similarity with the NH2 domain of the mammalian MLK. It also contains functionally conserved residues, and biologically has been shown to be an essential factor in fly embryo dorsal closure (Stronach and Perrimon, 2002).

The mammalian MLKs function as serine/threonine kinases and have been shown to regulate the MAP kinase cascades in a similar fashion to the MEKK group of proteins. Most importantly for this thesis, these proteins have been associated with JNK activation via activation of specific MKKs (Hirai et al., 1997; Merritt et al., 1999; Tibbles et al., 1996). The importance of MLK in JNK signaling was confirmed when members of the MLK family were implicated in JNK-mediated neuronal apoptosis (Xu et al., 2001). More recently, one member MLK3 has also been shown to play a role in the activation of both ERK and p38 (Chadee and Kyriakis, 2004).

The MLK subgroup contains 4 proteins characterised by highly conserved motifs including an amino SH3 domain, followed by a kinase domain, a leucine zipper and then CRIB domain (fig. 5). The leucine zippers are α-helices which have one face rich with leucines or any hydrophobic amino acid. These form coiled coils during MLK dimerisation by forming stable interactions with opposing hydrophobic leucine zippers. These leucine zippers are responsible for homodimerisation which are critical for activation of MLK (Leung and Lassam, 1998) and subsequent downstream JNK activation. The C terminal domains of this group diverge although all have a high proline content indicating possible

regulatory functions. The kinase domains share 75% sequence similarity and 65% homology between CRIB and SH3 motifs. The SH3 domains are universally thought to recruit MLKs to signaling and adaptor proteins that contain proline rich motifs. Studies have shown that disruption of the SH3 domain causes an increase in MLK activity (Zhang and Gallo, 2001). Therefore suggesting the SH3 motif negatively regulates MLK kinase activity, resulting in an MLK autoinhibitory function. MLK3 stands out from the other members in this group because it contains a Gly-Pro rich NH2 terminal domain. Information obtained about this family of proteins comes from the characterisation of MLK2 and MLK3.

MLK2 targets JNK through activation of MKK4 and/or MKK7. However, this protein has the ability to preferentially phosphorylate these MAP2Ks (Hirai et al., 1998). MLK2 has been shown to influence apoptosis induced by polyglutamine-expanded huntingtin protein and thus the JNK stress pathway (Liu et al., 2000c). JNK has the ability to target residues within the C terminal of MLK2 for phosphorylation during MLK2 activated apoptosis (Phelan et al., 2001). These data suggest that the ability of MLK2 to induce apoptosis lies within its C terminal region and it is this, which is targeted by JNK, possibly as a mechanism of feedback regulation. Similar phosphorylation sites were identified from phosphopeptide analysis of MLK3 (Leung and Lassam, 2001; Vacratsis et al., 2002). Within its C terminal domain 11 potential residues were found, seven of which contained proline immediately afterward suggesting these sites as possible targets for proline directed kinases or MAPKs. Further analysis could reveal these phosphopeptides in other MLK members.

Upstream kinases are able to regulate MLKs. For example, PAK1 can recruit MLK2 to an activated receptor via the adaptor protein NCK during activation of the JNK cascade (Poitras et al., 2003). Interestingly, it is clear MLK2 and MLK3 can interact with active Rac1 and Cdc42 via their CRIB domains (Nagata et al., 1998). In the same study MLK2 was shown to strongly activate JNK but this was not affected by co-expression of dominant negative Cdc42 or Rac1, suggesting activation of JNK is not dependent on MLK2 interaction with Cdc42. MLK2 also co-localised with active JNK1/2 in a patterned manner along microtubule structures. Further analysis revealed other binding partners of MLK2 including two members of the KIF3 family of microtubule motor proteins and KAP3 the target of the KIF3 motor complex. Other binding partners include clathrin, an essential protein in vesicle trafficking (Akbarzadeh et al., 2002). These data link stress signaling together with transport proteins identifying a possible mechanism by which specific signaling components can be localised to specific regions of the cell.

Cdc42 binds the MLKs via the CRIB domain (Bock et al., 2000) causing a conformational change. This opens the MLK protein and presents the leucine zipper making the protein available for homodimerisation. Although Cdc42 can induce the dimerisation and subsequent autophosphorylation of MLKs by disruption of SH3-autoinhibition, activation of downstream MKK4 is lost when the leucine zipper is disrupted (Vacratsis and Gallo, 2000), indicating the leucine zipper is essential to JNK activation, whereas Cdc42 is not.

MLK3 is the best characterised member of the MLK subgroup (Ing et al., 1994). It activates JNK via MKK4 and 7, as dominant negative experiments using

MKK4 and MKK7 provide protection against neuronal apoptosis induced by over expressed MLK3 (Xu et al., 2001). Alternatively, MLK3 activation in response to apoptosis can be inhibited by PKB leading to cell survival (Barthwal et al., 2003). As well as JNK, MLK3 has also been shown to activate the NF-κB pathway through phosphorylation of the IκB kinase complex (Hehner et al., 2000) and negatively regulate the cellular transformation properties of Rac1 (Lambert et al., 2002). Furthermore, MLK3 can also regulate B-Raf and ERK in order to control cell proliferation (Chadee and Kyriakis, 2004). Along with stress, agonists of MLK3 include ceramide, TNFα (Brancho et al., 2005; Sathyanarayana et al., 2002) and TGFβ (Kim et al., 2004) which all lead to the activation of JNK.

The Dual Leucine zipper bearing Kinases (DLKs) are composed of kinase domains, which are 87% identical, followed by 2 leucine-zipper motifs separated by a 31 amino acid spacer (fig. 5). DLK itself has a proline rich COOH term which is similar to the MLKs, but as yet no regulatory functions have been associated with this region (Holzman et al., 1994). As described earlier, DLK targets MKK7 but unlike MLK3, does not target MKK4 for phosphorylation to activate JNK (Merritt et al., 1999). MKK7 binds DLK through the leucine zipper domain (Mooney and Whitmarsh, 2004). DLK has also been shown to interact with the C terminal region of JIP-1 via its N terminal region and in doing so can regulate JNK activation (Mooney and Whitmarsh, 2004; Whitmarsh et al., 1998). DLK has been shown to play a role in presynaptic development as part of a p38 pathway regulated by the ubiquitin Ligase RPM-1 (Nakata et al., 2005) and differentiation (Robitaille et al., 2005). The other member of the group, LZK - leucine zipper kinase, only shows

catalytic domain homology with DLK (Sakuma et al., 1997) but also has two leucine zipper domains. In the same manner as DLK, LZK is able to autophosphorylate and target JNK for activation through MKK4, but unlike DLK not MKK7 (Ikeda et al., 2001). Both DLK and LZK have been found to associate with both plasma and cellular membranes suggesting a site for their activation and potential phosphorylation of substrates. Heterodimerisation has been observed between DLK and LZK through their amino termini, but not via the leucine zipper or through a direct interaction (Nihalani et al., 2000), indicating a possible function for an intermediate protein. A candidate for this function is the JNK-Interacting Protein-1 (JIP-1) (Nihalani et al., 2001). Most of the MLKs are able to interact with the JIP family and other scaffold proteins indicating the potential importance of these MLKs in regulating different pools of JNK under different responses (Ito et al., 1999; Kelkar et al., 2000; Yasuda et al., 1999).

The ZAK (leucine-zipper and sterile-α motif kinase) group are distinguished by the presence of a leucine zipper and sterile α motif (SAM) (Liu et al., 2000b) (fig. 5). These SAM domains are independently folded modules of approximately 70 amino acids usually found at either end of the protein and mediate homo- and heterodimerisation. Other proteins with these motifs include Receptor Tyrosine Kinases, adaptor and GTPase proteins. Preliminary data has indicated that ZAK mediates JNK signaling by activating MKK7 and not MKK4. This study also revealed an implication for ZAK in actin organisation and cellular morphology, as well as within cell cycle regulation (Yang, 2002).

This overview of the MAP3K members that activate the JNK cascade shows the sophisticated level of JNK regulation required for decisions of cell fate. The fact that many members are used in different responses suggests each pathway has to utilise a distinct pool of JNK to prevent any cross talk. Whether this segregation forms when cells are stimulated or these pools are maintained continually by potential scaffold proteins is yet to be answered. The relationship between components of the JNK pathway including JNK and scaffold proteins shall be discussed below.

1.9 Scaffold proteins

In order to function correctly, an important mechanism of JNK signaling is the organisation of upstream kinases into signaling complexes. This can be mediated by the interaction of the protein kinases with one member of the cascade (Xu and Cobb, 1997) or alternatively, scaffold proteins have been shown to also mediate these JNK signaling pathways (Morrison and Davis, 2003). Scaffold proteins increase the local concentration of pathway components thereby increasing the chance a signal can be transmitted. In binding MAPK members through distinct docking domains, scaffold proteins can maintain signal specificity. Furthermore, by interacting with other proteins, for example kinesin, scaffold proteins can localise MAPK pathways to specific regions within the cell. One other important advantage of utilising scaffold proteins is that they insulate active MAPK components from other signals therefore preventing unwanted crosstalk with other MAPK modules.

This allows the precise regulation of the MAPK, which is essential in an environment where so many signals are continuously processed.

In *Saccharomyces cerevisiae* scaffolds have been shown to be physiologically important. The pathway controlling the mating response using Ste11p-Ste7-Fus3p uses the scaffold protein Ste5p (fig. 6), which has distinct binding sites for all of the kinases (Printen and Sprague, 1994). Genetic analysis indicates that Ste5p can co-operate with these kinases to enhance signaling within this system. The Hog1p osmosensing pathway is also activated by Ste11p, but this pathway utilises a different scaffold protein, Pbs2p (Posas and Saito, 1997). Ste5 associates with the G protein subunit βγ (Whiteway et al., 1995) and this interaction allows the Ste5 scaffold to localise MAPK signaling to a specific environment (Pryciak and Huntress, 1998). For example, Ste5 can shuttle to and from the nucleus (Mahanty et al., 1999) and under activation in the pheromone response accumulate at the tips of mating projections (van Drogen et al., 2001). These pathways have been well characterised (Park et al., 2003) and represent an important example of how scaffold proteins are used by MAPKs to function in multiple responses.

1.9.1 CrkII and filamin

Possible mammalian scaffolds include CrkII, an adaptor molecule also known to associate with tyrosine phosphorylated p130Cas and paxillin. CrkII binds JNK1 through a proline rich region of JNK1 and the N terminal SH3 domain of CrkII. This interaction specifically controls JNK activation in response to epidermal growth

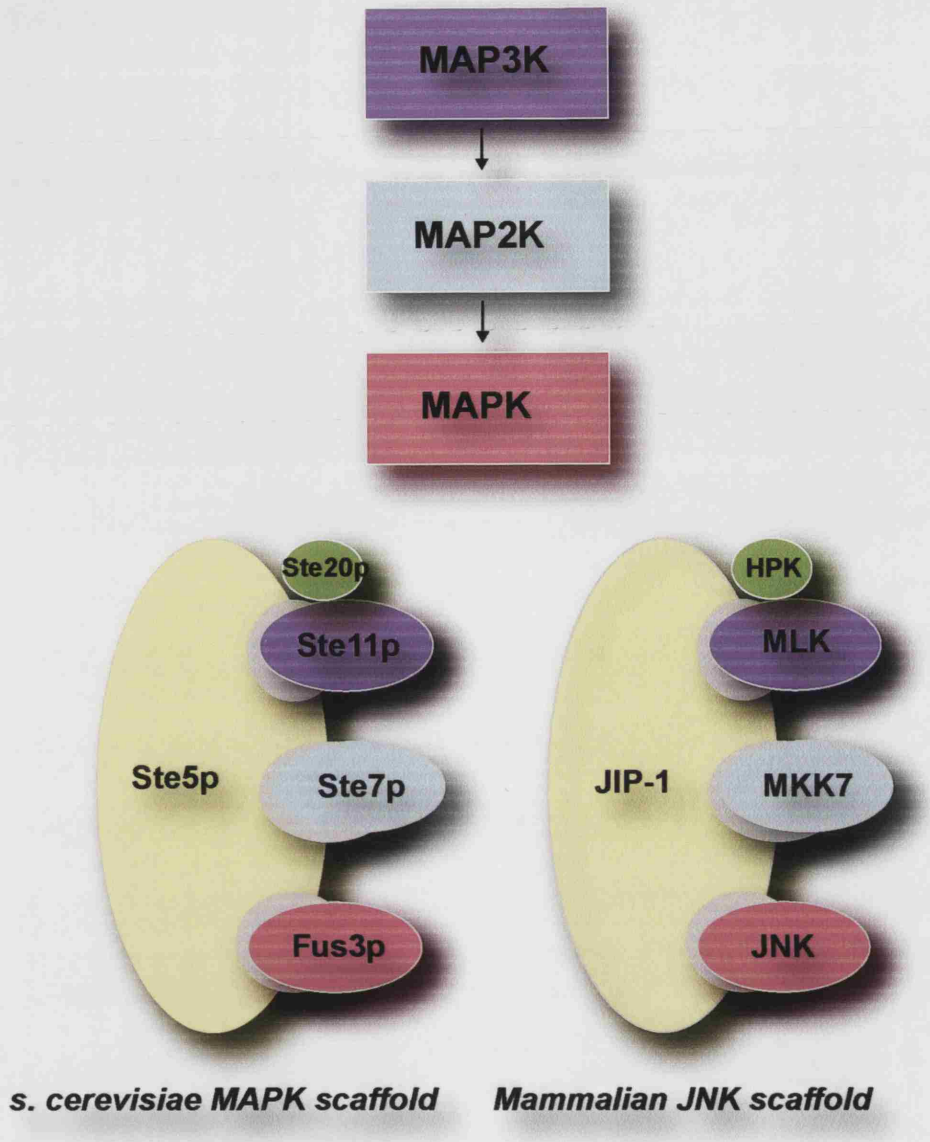

Figure 6 – 1.9 Yeast and mammalian MAPK scaffold complexes

Top panel - Schematic diagram representing the passage of a signal through a MAPK cascade.

Bottom panel – Schematic diagram representing the MAP3Ks, MAP2Ks and MAPKs which interact with S. cerevisiae and mammalian scaffold proteins, Ste5p and JIP-1, respectively. Each colour represents which group of MAPK each protein belongs to in reference to the top panel. These scaffolds bring together the components of the pathway to enhance the output. In yeast the Ste5p scaffold is essential for the function of this MAPK pathway.

factor through Rac-1. CrkII forms a complex with p130Cas where it is thought JNK is activated by the presence of HPK1 and MKK4 (Girardin and Yaniv, 2001).

Filamin is a large actin-binding protein, which has also been shown to bind MKK4 (Marti et al., 1997). This study showed human melanoma cells which lack filamin were unable to activate JNK via the TNF pathway. This indicates filamin is an important factor in JNK signaling under TNF receptor activation in this cell type. Filamin has also been shown to interact with the zinc finger domain of the adaptor protein TRAF2 (Leonardi et al., 2000). As mentioned earlier this protein is recruited to the TNF receptor under activation and has a key role in JNK activation by TNFα. Therefore filamin has a potential role as a scaffold protein to coordinate TNFα-activated JNK signaling via TRAF2 and MKK4.

1.9.2 POSH

The POSH (Plenty Of SH3s) protein is a zinc finger protein that contains four SH3 domains. Initial studies demonstrated POSH was involved in apoptosis associated with activation of NF-κB and JNK (Tapon et al., 1998). A more recent study indicated that POSH can act to scaffold members of the neuronal apoptotic JNK pathway including activated Rac-1, JNK, MKK4 and MKK7 and some members of the MLK group including MLK2, MLK3 and DLK (Xu et al., 2003). Using RNAi to reduce the expression of POSH, led to decreased JNK activation and apoptosis in PC12 cells induced by NGF deprivation. This work identified a physiological role for POSH as the JNK scaffold protein required for JNK-dependent neuronal apoptosis (Xu et al., 2003). In addition, it has been suggested the interaction

between MLK3 and POSH can be negatively regulated by Akt (Figueroa et al., 2003). They suggest through phosphorylation of MLK3 by Akt the multiprotein JNK signaling complex is disassembled; therefore leading to inhibition of JNK signaling.

1.9.3 SKRP1

SAPK pathway-regulating phosphatase-1 (SKRP1) (also known as JKAP) and JNK stimulatory phosphatase-1 (JSP-1) (also known as MKP-X) are MAPK phosphatases (MKPs) which have the ability to inactivate JNK through dephosphorylation (Alonso et al., 2002; Zama et al., 2002a). However, unusually they also have the ability to activate JNK and to act as scaffolds for members of the JNK cascade, MKK7 and ASK1 (Alonso et al., 2002; Chen et al., 2002; Shen et al., 2001; Zama et al., 2002a; Zama et al., 2002b). The significance and mechanism of this function of MKPs as scaffold proteins remains unclear.

1.9.4 The JIP family of proteins

The JNK Interacting Proteins are an important group of proteins, which have been shown to interact with members of the JNK pathway. Initially JIP-1 was characterised using a two-hybrid screen as an inhibitor of the JNK pathway and thus JNK induced gene expression (Dickens et al., 1997). JIP-1 was shown to bind to both JNK1 and JNK2 but not ERK or p38. Interestingly, the affinity of JNK2 for JIP-1 is 100-fold greater than that for the JNK substrate c-Jun. Subsequently three other

members have been discovered. The proteins can be separated into two groups dependent on sequence similarity. JIP-1 and JIP-2 are very similar in sequence and are ubiquitously expressed. They contain SH3 and PTB domains present in the C terminal region (Yasuda et al., 1999) (fig. 7). The extreme C terminal region is highly conserved and interacts with the tetratricopeptide repeat (TPR) motif within the light chain of kinesin (KLC) (Verhey et al., 2001). Kinesin is a motor protein, first described in squid axonal transport, which uses energy from ATP hydrolysis to transport vesicles and organelles along microtubules. The link between these two proteins is consistent in that the JIP proteins generally localise to the tips of neuronal cells which places them at the plus end of microtubules and therefore in the correct orientation for microtubule transport. Genetic analysis confirms this link, as deletions within JIP-1 abolish the interaction with the KLC as well as the localisation of JIP-1 to the nerve terminal (Verhey et al., 2001). This indirect relationship between cellular transport and the JNK signaling module is an attractive concept as it allows scaffolding proteins to assemble multiprotein complexes and under stimulation transport these modules to a specific location. For example, under stress JIP-1 moves from the neurites to the soma of hippocampal cells where it co-localises with activated JNK (Whitmarsh et al., 2001). This interaction with kinesin suggests the potential ability of JNK to phosphorylate kinesin in order to activate cellular transport.

The PTB domains of JIP-1 and -2 interact with p190 RhoGEF (Meyer et al., 1999), amyloid precursor protein (Matsuda et al., 2001), and apolipoprotein E receptor 2 (Stockinger et al., 2000). These proteins therefore have the potential to be

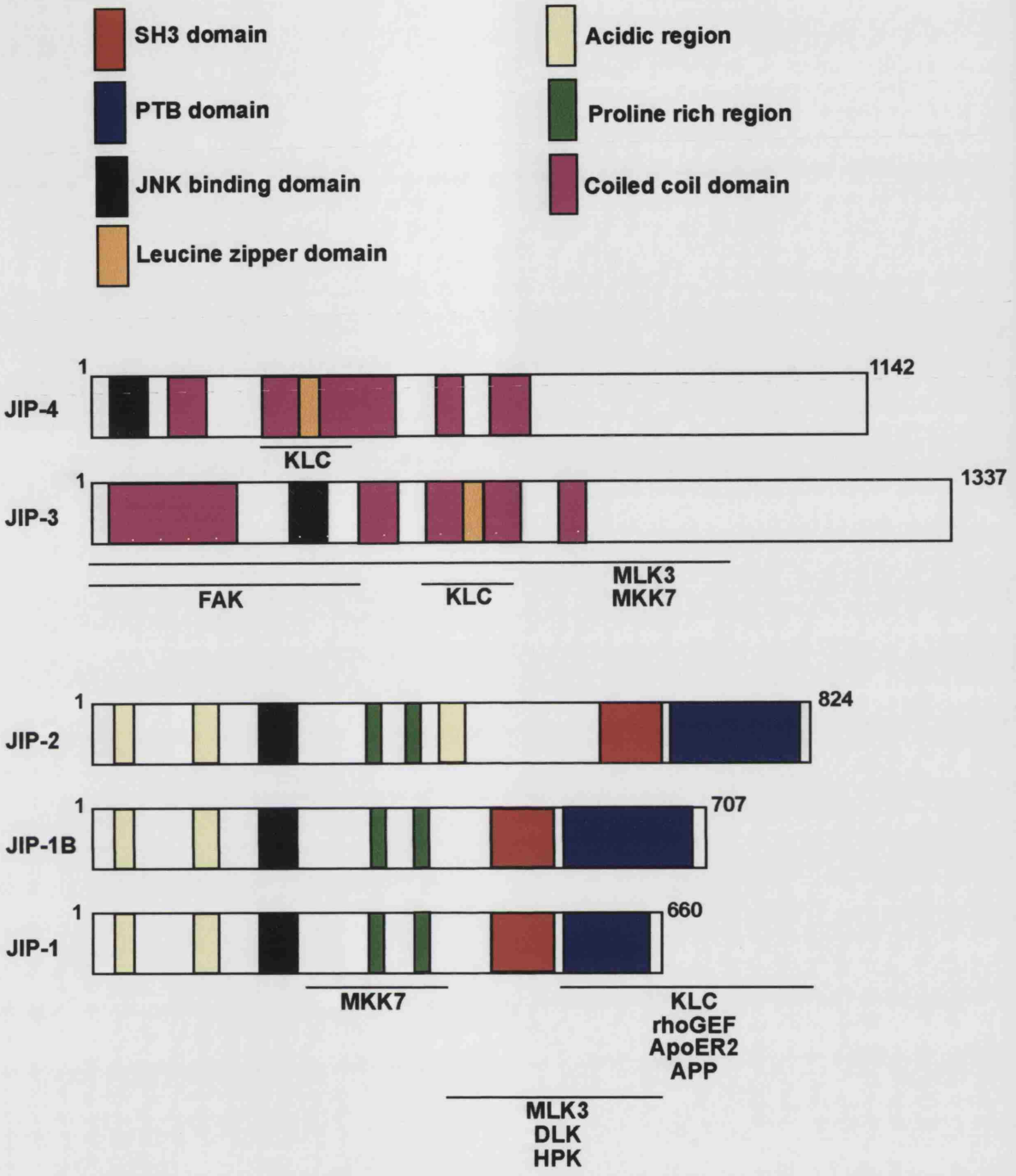

Figure 7 – 1.9.4 The JNK interacting proteins (JIPs)

Cartoon illustrating domains present in the four members of the JIP family of proteins (names on left). A coloured key is provided to identify each domain. *Numbers* refer to amino acid position. Proteins which interact with the JIPs and their positions are also shown.

transported via JIP-1 and kinesin. JIP-1 accumulates in the growth cones of the developing brain. New data suggest in this location JIP-1 links kinesin, JNK and DCX, a protein essential for neuronal migration in the developing brain. This leads to phosphorylation of DCX by JNK, failure of which results in affected neurite outgrowth (Gdalyahu et al., 2004).

JIP-1 associates with the other components of the MAPK module, MKK7, MLK3, DLK and the Ste20-related protein kinase, HPK (fig. 6) (Whitmarsh et al., 1998) through distinct regions (fig. 7). This association with JIP-1 lead to the enhanced activation of JNK. JIP-1 is thought to pre-assemble this multiprotein complex, binding only a small proportion of the population of each component as these levels change very little under activation by stress (Whitmarsh et al., 2001). Analysis of binding between JIP-1 and MAPK components has identified separate binding domains for JNK, MKK7 and MLK members as well as residues in JNK responsible for its interaction with each of these proteins including JIP-1 and its substrate c-Jun (Mooney and Whitmarsh, 2004; Whitmarsh et al., 1998) (fig. 7). Whether all the components simultaneously interact with the JIP-1 is not clear. However, analysis of binding indicates functional interactions between the proteins bound to JIP-1 i.e. the binding of JNK reduces the affinity of JIP-1 for MLK and DLK (Nihalani et al., 2001; Nihalani et al., 2003). In addition, Akt, with a similar mechanism to the POSH scaffold, binds JIP-1 and in doing so prevents activation of the JNK pathway; this interaction also enhances the activation of Akt (Kim et al., 2003; Kim et al., 2002).

JIP-2 has also been shown to bind components of the MLK group of proteins as well as MKK7, and JNK2 (Yasuda et al., 1999) as well as MKK3, p38α and p38δ (Buchsbaum et al., 2002; Schoorlemmer and Goldfarb, 2002). These interactions can lead to the activation of p38α in response to Rac exchange factors Tiam1 and Ras-GRF1 and p38δ under increased expression of Fibroblast growth factor homologous protein family (FHF). Both these proteins selectively enhance JNK activation under MLK signaling but do not associate with the MEKK proteins or enhance ERK activation. Interestingly both JIP-1 and JIP-2 form homo- and hetero-oligomeric complexes, thus aggregating multiple components of the JNK signaling pathway, possibly to enhance signal potential but also to insulate the pathway against non-specific signaling. These data suggest JIP-1 and JIP- 2 act as molecular scaffolds to mediate JNK signaling pathways.

JIP-3 (also known as JSAP1) and JIP-4 (also known as JLP) are related proteins that are distinct from JIP-1 and JIP-2 in both structure and sequence. These proteins contain coiled-coil and leucine zipper domains (Ito et al., 1999; Kelkar et al., 2000; Lee et al., 2002) and have been shown to also bind the KLC via these coiled-coil and leucine zipper domains in their NH2 terminal domains (Bowman et al., 2000; Kelkar et al., 2005; Lee et al., 2002). The domain of JIP3 which binds KLC is highly homologous to the *Drosophila* Sunday Driver Protein (SYD). Genetic studies of *Drosophila* discovered that various cellular axonal transport using kinesin is dependent on SYD (Bowman et al., 2000). It acts as a scaffold, by interacting with the KLC, carrying post golgi vesicles along microtubules to the axons of neurons. A homolog of JIP3 has also been identified in *C.elegans* which also regulates the

location of vesicular cargo by integrating JNK signaling (Byrd et al., 2001). JIP-3 also enhances JNK activation through binding JNK, MKK7 and members of the MLK group (Kelkar et al., 2000) and potentially MEKK1 and MKK4 (Ito et al., 1999) and Fak (Takino et al., 2002), whereas JIP-4 has been shown to bind MKK4 and MEKK3 (Lee et al., 2002). JIP-3 also binds the ASK1 MAP3K in response to hydrogen peroxide resulting in ASK1 phosphorylation of JIP-3. This phosphorylation recruits JNK3 and MKK4/7 leading to the activation of JNK (Matsuura et al., 2002). Interestingly JIP3 has also been shown to associate with Toll-Like Receptor 4 (TLR4) involving JIP3 in LPS-mediated JNK activation (Matsuguchi et al., 2003) and a potential physiological role. Even though JIP-4 can scaffold some MAPK members and binds JNK, it is unable to activate JNK signaling but through interactions with MKK3 and MKK6, JIP-4 can potentiate p38 activation (Kelkar et al., 2005).

Although it is established that the JIPs do enhance the activation of JNK by specific stimuli, the physiological role for JIP is still relatively unclear. Studies performed on mice lacking JIP-1 demonstrated defects in stress-induced JNK activation in neurons following excitotoxic shock (Whitmarsh et al., 2001). Further data suggests JIP-1 aids the phosphorylation of the adaptor protein IRS-1 by JNK which leads to an inhibition of the insulin receptor (Jaeschke et al., 2004). This confers a cellular resistance to the effects of insulin and therefore a potential role in the development of type 2 diabetes. Further possible roles included are in cytokine induced apoptosis (Haefliger et al., 2003) and in the pathology of Alzheimer's disease (Scheinfeld et al., 2003). Mice lacking JIP-3 were non viable, however cellular analysis identified severe defects in the telencephalic commissure (the

connection between the left and right hemispheres of the brain) (Kelkar et al., 2003). The first instance of complementation between the JIP family members was shown by the partial rescuing of this JIP-3 neuronal defect by expression of transgenic JIP-1 (Ha et al., 2005).

1.9.5 β-arrestins

In mammals the arrestin family consists of 4 members: two visual arrestins (rod and cone) and β-arrestin 1 and -2. The visual arrestins are expressed mainly in the retina whereas the β-arrestins are widely expressed although particularly in the CNS, heart and testes. This family of proteins are highly conserved through evolution and homologs exist in both *Drosophila* and *C. elegans*. The visual arrestins were initially discovered as inhibitors of rhodopsin signaling (Wilden et al., 1986) and subsequent work led to the discovery of the β-arrestins (Attramadal et al., 1992; Lohse et al., 1990). Through analysis of the crystal structure of visual arrestin these proteins were shown to consist of 2 domains each made up of a seven stranded β sandwich (Hirsch et al., 1999). The crystal structure of β-arrestin 1 is shown in figure 8a. The N and C domains are connected by a flexible linker containing a phosphate sensor, which, along with separate sites within the C terminal domain, is thought to regulate the conformation of these proteins. For example, β-arrestin 1 relies on phosphorylation at specific residues within its C terminal domain to bind clathrin and thus aid internalisation. Furthermore, analysis of tryptic proteolysis patterns shows how β-arrestin 2 undergoes a conformational change when bound by active phosphorylated GPCR which releases the C terminal tail enhancing its

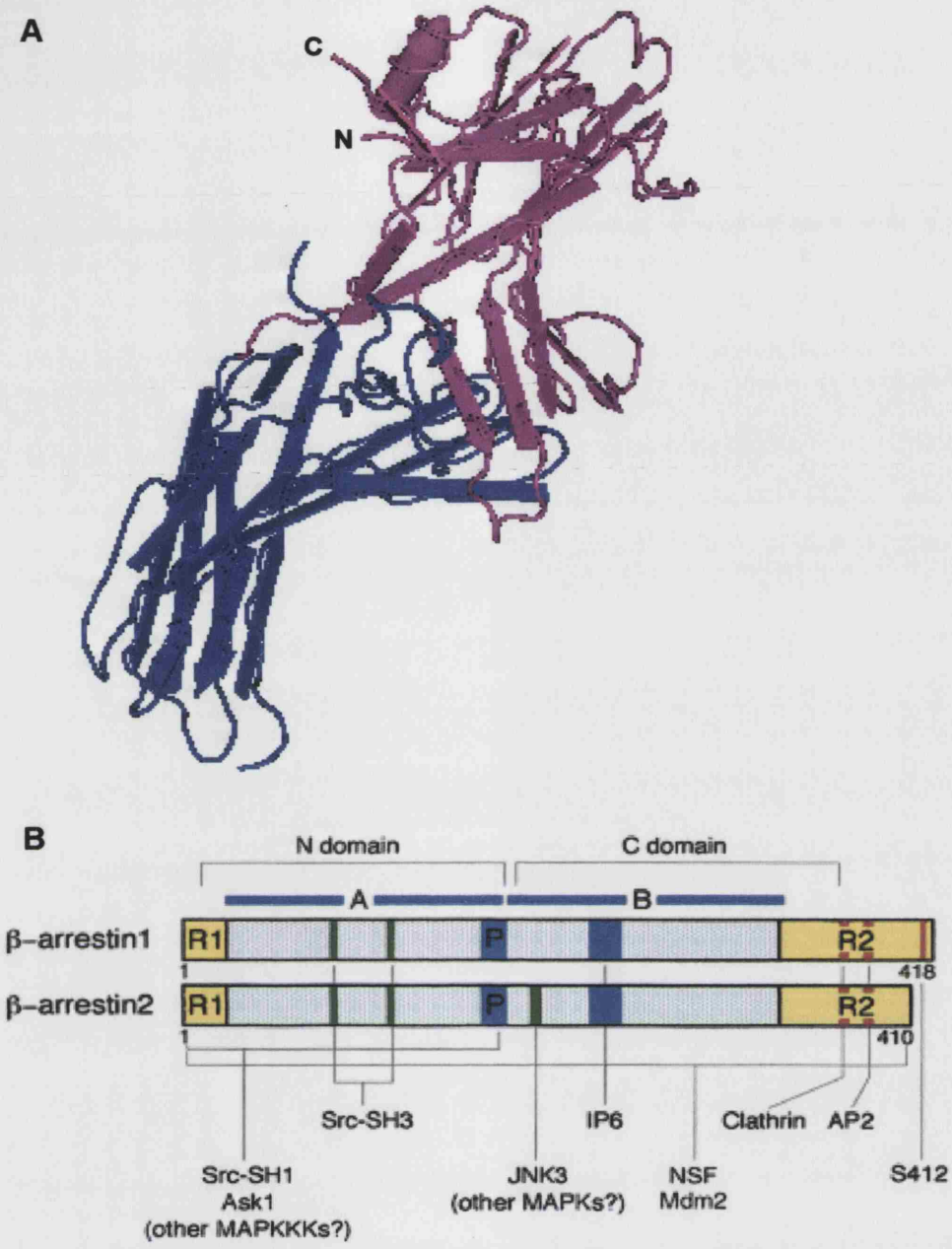

Figure 8 – 1.9.5 beta-arrestins

A – Crystal structure of bovine beta-arrestin 1 made using CnD3. These proteins consist of two domains; N domain (*pink*) and C domain (*blue*). The tails of the protein are represented by *N* and *C* indicating their close proximity.

B – Cartoon representing the domain structure of the beta-arrestins. *Numbers* refer to amino acid positions; *N* and *C* indicate each domain; *A* and *B* represent major functional domains; *P* refers to the phosphatase sensor domain; *R1* and *R2* are regulatory domains; *blue* represents sites involved in receptor or membrane interactions; *green* shows interactions with signaling proteins; *red* shows interactions with the endocytic machinery; *S412* phosphorylation site.

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ability to bind clathrin by 10-fold (Xiao et al., 2004). β-arrestin 2 exhibits 78% amino acid homology with β-arrestin 1 but contains an insertion in the C tail which has been implicated in clathrin binding but may also hold other specific regulatory functions. Both proteins function in the desensitisation and internalisation of most G-protein coupled receptors (GPCRs) (Ferguson et al., 1996). The domain architecture of the β-arrestins is shown in figure 8b.

GPCRs are a family of 7-transmembrane spanning protein receptors widely expressed and involved in extracellular to intracellular signal transmission. These receptors are activated by a wide variety of ligands including growth factors, hormones, light, and peptide & non-peptide neurotransmitters. For example, the angiotensin type 1a receptor (AT1aR) is activated by the hormone angiotensin II. Ang II plays an important role in the control of blood pressure through its actions on vascular smooth muscle contractibility, aldosterone secretion, ion transport and dipsogenic responses in the brain. The triggering of AT1aR by Ang II results in a conformation change that allows binding of G proteins and which leads to an increase in the GTPase activity of bound heterotrimeric G proteins (α, β and γ). Downstream signaling from these receptors through adenylyl cyclases, phospholipases and ion channels depends on the sub-type mix of G proteins present. Activated GPCRs are also substrates for G-protein coupled receptor kinases (GRKs) and second messenger related kinases (PKA and PKC). The latter are able to phosphorylate both active and inactive receptors leading to heterologous desensitisation, whereas GRKs target ligand-associated receptors and are able to discriminate between active and inactive GPCRs resulting in homologous

desensitisation. Furthermore, GRKs phosphorylate active GPCRs in specific regions within the C terminal tail and third intracellular loop of the receptor. β-arrestin 2 recognises both the conformational change and GRK phosphorylation of the receptor and translocates from the cytoplasm to bind the GPCR tail. This interaction physically blocks the binding of G proteins to the receptor and thus terminates any downstream signaling resulting in desensitisation of the GPCR (Luttrell and Leffkowitz, 2002).

β-arrestins are also responsible for the internalisation of GPCRs. They act as adaptor proteins mediating the formation of complexes between receptor and specific proteins required for endocytosis (figure 9a). These include the heavy chain of clathrin via LIEL/F motif (Goodman et al., 1996; Krupnick et al., 1997); AP-2, essential for the formation of clathrin-coated pits (Laporte et al., 1999); NSF, an ATPase critical for intracellular membrane trafficking (McDonald et al., 1999); Ral-GDS, dissociation with β-arrestin 2 stimulates cytoskeletal rearrangements (Bhattacharya et al., 2002); and ARF6, G protein involved in the endocytosis process (Claing et al., 2001). Sites for some of these proteins have been identified within the structure of β-arrestins and are shown in figure 8b. In response to agonist binding β-arrestin 2 also undergoes rapid ubiquitination by Mdm2 which is essential for receptor internalisation and may play a role in receptor sorting and degradation in the lysozyme (Shenoy et al., 2001). The clathrin-coated vesicles containing the receptor and β-arrestin are internalised and become endosomes. Due to their acidic nature these endosomes cause the destruction of the ligand and thus a conformational change in the receptor. It is thought a phosphatase then

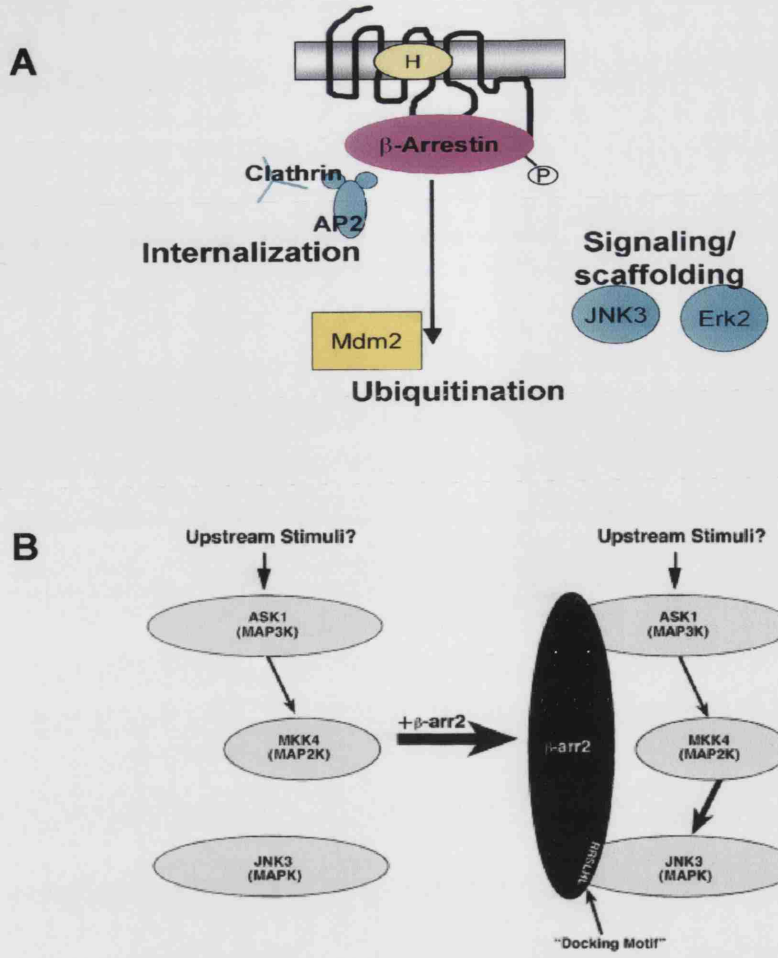


Figure 9 – 1.9.5 beta arrestin 2: Role in desensitisation, internalisation and as a scaffold protein

A – Schematic diagram representing the roles and interactions of beta arrestins. Activation of GPCRs leads to specific recruitment of beta arrestins to the C terminal tail of the receptor. This interaction leads to desensitisation and internalisation of the GPCR by beta arrestins through interactions with clathrin, AP-2, NSF, Ral-GDS and ARNO. In response to agonist binding beta arrestin 2 undergoes ubiquitination by Mdm2, a process essential for receptor internalisation. In addition beta arrestins can act as signal transducers by binding to signaling components including Src, ERK and JNK3 cascades.

B – Schematic diagram representing interaction of beta arrestin 2 with members ASK1, MKK4 and JNK3 of the MAPK cascade indicating the presence of beta-arrestin 2 enhances the ability to MKK4 to activate JNK3.

Diagrams A and B are exerts from Shenoy and Lefkowitz (2003) Biochemical Journal and Miller et al (2001) The Journal of Biological Chemistry, respectively.

dephosphorylates the C terminal tail allowing the dissociation of β-arrestin. Depending on the GPCR type, through an unknown mechanism, the receptor is either recycled to plasma membrane or targeted for degradation (Shenoy and Lefkowitz, 2003).

The critical role of β-arrestins in desensitisation and internalisation were confirmed from knockout mice of β-arrestin 1 and -2. Mice lacking β-arrestin 1 were normal except responded with a hyperactive cardiac function in response to adrenaline due to impaired β-adrenergic receptor desensitisation (Conner et al., 1997). Whereas mice minus β-arrestin 2 exhibited a prolonged analgesic effect of morphine suggesting that desensitisation of the GPCR μ-opiod receptor (μOR) was impaired (Bohn et al., 1999). Further work showed that β-arrestin 2-induced desensitisation of μOR is responsible for tolerance to morphine but not the physical dependence associated with addiction (Bohn et al., 2000). As well as in the CNS, β-arrestin 2 also aids endocytosis in development through Fizzled 4 7MSR via Dishevelled 2 (Chen et al., 2003), and Smoothened 7MSR (Chen et al., 2004; Wilbanks et al., 2004)

In addition to their role in receptor inhibition, β-arrestins are also signal transducers and have been shown to interact with intracellular signaling components including both tyrosine and MAP kinases. For example, β-arrestins have been shown to recruit the Src family kinases (Miller et al., 2000) to endothelin ETa receptors (Imamura and al, 2001), neurokinin 1 receptors (DeFea et al., 2000) and CXCR1 receptors (Barlic and al, 2000) resulting in the activation of the MAPK ERK1/2 (Luttrell et al., 1999). β-arrestin 2 has also been shown to directly recruit

components of the ERK MAPK signaling module to the AT1aR (Luttrell et al., 2001). While G protein dependent ERK signaling is responsible for activation of transcription factor EGR-1 after AT1aR stimulation, the β-arrestin 2 bound pool of ERK is retained in the cytoplasm, targeting activated ERK to cytoplasmic substrates (Pearson et al., 2001; Wei et al., 2004). β-arrestin 2 was also recently reported to bind JNK2 and JNK3, and upstream components ASK1, and indirectly MKK4, to stimulate JNK3 activation through AT1aR stimulation (McDonald et al., 2000; Miller et al., 2001). Sites on β-arrestin 2 for known signaling proteins are shown in figure 8b. It is unclear whether MKK4 interacts with β-arrestin 2 and ASK1 or JNK3; however it is possible a simultaneous interaction would stimulate MKK4 to assemble into the complex (fig 9b). This scaffold complex brings JNK activation under the control of GPCR signaling as formation of this complex selectively activates the JNK3 isoform. Similarly to ERK, JNK may bind β-arrestin 2 to maintain an extra nuclear location near to specific cytosolic substrates (Scott et al., 2002).

These scaffold proteins all have the ability to bind JNK and specific upstream kinase components. Importantly, they can recruit JNK modules to specific regions of the cell where JNK can be activated and controlled by specific stimuli i.e. β-arrestin 2 brings JNK3 signaling under the control of GPCR activation. Many of these scaffolds retain JNK in the cytoplasm for a period of time thus preventing translocation to the nucleus where classically JNK activates substrate transcription factors and is also dephosphorylated by protein phosphatases. However, evidence suggests scaffolds enhance JNK activity resulting in an increase in c-Jun activation. Therefore, activated JNK may be released after its accumulation on scaffolds to

translocate to the nucleus. However, during the scaffold-bound period these scaffolds may maintain JNK in an active state i.e. away from nuclear phosphatases, so it can target cytosolic substrates or to prevent JNK from activating its nuclear substrates. Whether JNK is negatively regulated by protein phosphatases when associated with scaffold proteins in the cytoplasm is unclear. The issues surrounding the negative regulation of JNK by protein phosphatases shall be discussed below.

1.10 Negative regulation of MAPKs by protein phosphatases

In contrast to the regulation of JNK by upstream kinases, little is understood about the physiological negative regulation of JNK. The process of JNK activation by phosphorylation on threonine and tyrosine residues is reversible, indicating phosphatases as the obvious candidates for JNK negative regulation. The addition of general cellular phosphatase inhibitors cells induces massive JNK activation. The duration and extent of MAPK activation could therefore be governed by the balance between the activity of the MAPKK and protein phosphatase. There are two main types of cellular phosphatases; tyrosine-specific phosphatases (for example PTP1B) and serine threonine phosphatases (for example PP2C). As suggested, tyrosine phosphatases target substrates with phospho-tyrosine residues, whereas serine threonine phosphatases dephosphorylate either phospho-serine or phospho-threonine residues of their substrates. MAPK can be inactivated either through dephosphorylation of tyrosine and/or threonine residues of the T-X-Y motif. Therefore threonine serine phosphatases (Alessi et al., 1995; Morita et al., 2001;

Takekawa et al., 1998), and protein tyrosine phosphatases (Pulido et al., 1998; Saxena et al., 1999) are able to negatively regulate the different groups of MAPK. Alongside these exist a family of dual specificity phosphatases (DSPs)(MAP Kinase Phosphatases (MKPs)) which are able to dephosphorylate both the tyrosine and threonine residues present in the activation motif T-X-Y of MAPK (Sun et al., 1993).

1.10.1 Regulation of MAPK signaling by phosphatases in *Saccharomyces cerevisiae* and *Drosophila melanogaster*

In *S. cerevisiae* different groups of protein phosphatases have been shown to act in coordination to regulate Fus3, the MAPK responsible for activating the mating response. Inactivation of Fus3 is required in order for the cell to re-enter a normal growth phase. Candidate phosphatases are the protein tyrosine phosphatases PTP2 and PTP3 as they have been shown to negatively regulate stress signaling in *S. cerevisiae* (Wurgler-Murphy et al., 1997). The study identified the tyrosine phosphatase, PTP3, as the phosphatase which controls the low basal level activation of Fus3 (Zhan et al., 1997). However, PTP3 works together with dual specificity phosphatase MSG5 to control Fus3 activation after pheromone stimulation (Doi et al., 1994). Furthermore, MSG5 expression is induced during the Fus3 activated pheromone response by the transcription factor Ste12p, indicating DSPs can function as part of a negative feedback system of regulation. The interaction between PTP3 and Fus3 uses a CH2 (Cdc25 homology) domain which is conserved among many cellular phosphatases including dual specificity phosphatases. Mutations which abolish this interaction have resulted in the dysregulation of Fus3

(Zhan and Guan, 1999). This indicates that the direct interaction between PTP3 and Fus3 is essential for the negative regulation by the phosphatase. MSG5 and Sdp1 are dual specificity phosphatases that have also been shown to regulate the Slt2 kinase, the MAPK which triggers a response under changes in cell wall integrity (Flandez et al., 2004; Hahn and Thiele, 2002). It has been suggested MSG5 interacts with and dephosphorylates Slt2. However, under activation of the cell integrity pathway, Slt2 is able to phosphorylate MSG5 leading to a decrease in binding between the two proteins. This identifies a mechanism under which phosphatases can regulate MAPKs through specific interactions.

During the mating response both Fus3 and KSS1 (which also signals in the invasive growth pathway) are activated by the same Ste5p scaffold system. However, KSS1 activation is abrogated by activated Fus3 as a mechanism of preventing cross talk with the other KSS1 pathway (Sabbagh et al., 2001). Furthermore, MSG5 works in conjunction with Ste5p in order to differentially regulate the MAPKs, KSS1 and Fus3 mating response (Andersson et al., 2004). These data identify a relationship between a MAPK scaffold and DSP which is essential in controlling the physiological response.

Within the *Drosophila* genome six DSPs have been identified including puckered and DMKP-3. The target MAPK for puckered MKP is the *Drosophila* equivalent of JNK, DJNK, which has a role in regulating cell differentiation and morphogenesis during dorsal closure (Martin-Blanco et al., 1998). Mutations in the puckered gene lead to the hyperactivation of DJNK and over expression of puckered results in a phenotype similar to that of cells lacking DJNK. Interestingly,

puckered expression is induced through DJNK activation during dorsal closure resulting in a negative feedback mechanism.

Furthermore, DSPs have also been identified in *Arabidopsis thaliana* (Ulm et al., 2001). In this study, mutational analysis identified a MKP as having the ability to regulate the MAPK response to genotoxic stress and in doing so maintain cell viability. In *C. elegans* a homolog to a mammalian MKP, VHP-1, has been shown to regulate the p38 homolog, PMK-1, during the immune response and defence against pathogens. One of the first DSPs to be identified was VH1 a gene expressed in late stage viral infection by vaccinia virus (Guan et al., 1991). The conservation of these DSPs and their specific function in many species confirms they have a key role in MAPK regulation and are an important model for their mammalian cousins.

1.10.2 Mammalian dual specificity phosphatases

In mammalian cells ten classical DSPs have been identified as MKPs with the ability to selectively hydrolyse phospho-Thr and phospho-Tyr residues on activated MAPKs thereby inactivating the MAPK. The MKP gene family have been isolated from various organisms resulting with somewhat confusing nomenclature which are described in figure 10. The first mammalian MKP was cloned from the mouse as MKP-1 and later its human homolog was also identified, CL100 (Charles et al., 1992; Keyse and Emslie, 1992). This phosphatase is rapidly induced by oxidative stress, growth factors, heat shock, UV, LPS and anisomycin and can dephosphorylate specific members of the p38, JNK and ERK MAPK families

DSP		Subcellular localisation	MAPK substrate preference
MKP-1(m)/CL100(h)	(DUSP1)	Nuclear	p38, JNK >> ERK
PAC1(h)	(DUSP2)	Nuclear	ERK >> p38, JNK
VHR	(DUSP3)	Nuclear	ERK >> JNK, p38
MKP-2(r)/VH2(h)	(DUSP4)	Nuclear	ERK, JNK, p38
VH3(h)/B23	(DUSP5)	Nuclear	ERK, JNK, p38
MKP-3(r)/PYST1	(DUSP6)	Nuclear/Cytosolic	ERK >> JNK, p38
MKP-X(r)/PYST2	(DUSP7)	Cytosolic	ERK, JNK, p38
MKP-4/PYST3	(DUSP9)	Nuclear/Cytosolic	ERK, p38, JNK
MKP-6	(DUSP13)	Cytosolic	ERK, JNK, p38
M3/6(m)/hVH5	(DUSP8)	Nuclear/Cytosolic	JNK, p38 > ERK
MKP-5	(DUSP10)	Nuclear/Cytosolic	JNK, p38
MKP-7	(DUSP16)	Nuclear/Cytosolic	JNK, p38 > ERK

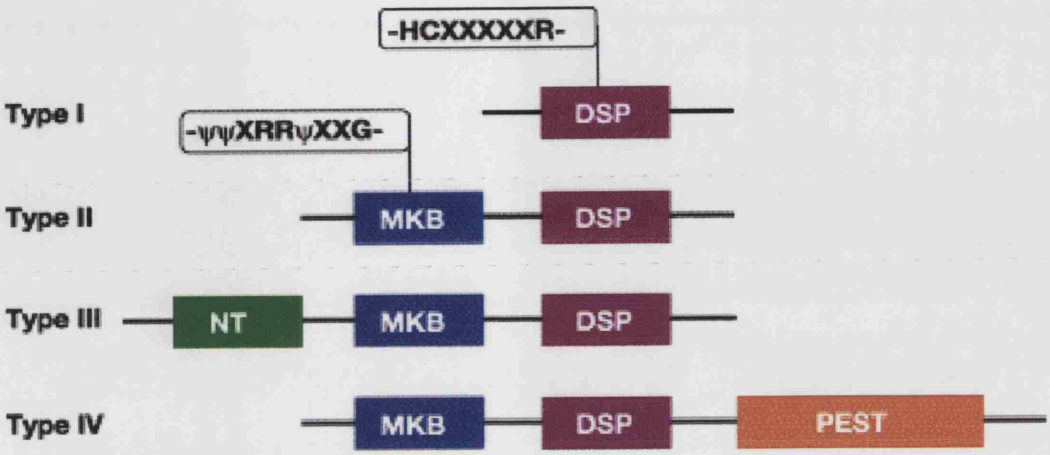
Figure 10 - 1.10.2 Dual Specificity Phosphatases (DSPs)

A table compiling details of the known mammalian dual specificity phosphatases. Alternative names are for each protein in human (h), rat (r) or mouse (m). The cellular location of these phosphatases is also shown, along with their MAPK substrate specificity.

(Alessi et al., 1993). However, it is clear this induction and subsequent choice of substrate MAPK is dependent on cell type and the ability of CL100/MKP-1 to target numerous MAPKs is dependent on specific protein-protein interaction domains (Slack et al., 2001). The family of DSPs has grown from then on and includes VHR (Ishibashi et al., 1992), PAC1 (Rohan et al., 1993), MKP-2/VH2 (Guan and Butch, 1995; Misra-Press et al., 1995), VH3/B23 (Ishibashi et al., 1994; Kwak and Dixon, 1995), MKP-3/PYST1 (Groom et al., 1996), MKP-X/PYST2/JSP1 (Alonso et al., 2002; Shin et al., 1997), VH5/M3/6 (Martell et al., 1995; Theodosiou et al., 1996), MKP-4/PYST3 (Muda et al., 1997), MKP-5 (Tanoue et al., 1999) and MKP-6 (Marti et al., 2001), MKP-7 (Masuda et al., 2001; Tanoue et al., 2001b). These proteins share critical common features, all have sequence homology to the VH1 DSP described earlier and selectively dually dephosphorylate different MAPKs as shown in figure 10.

1.10.2.1 DSP catalytic function, structure and regulation

The family of DSPs can be split up into subtypes depending on their domains as shown in figure 11. General domains include, the less conserved amino/rhodanese terminal domain containing two short regions, that are homologous to the cell regulator Cdc25 phosphatase (Keyse and Ginsburg, 1993), and a cluster of basic residues; both of these sites are important for protein-protein interactions including MAPK docking domains (MKB) (Muda et al., 1998; Tanoue et al., 2000). The corresponding MAPK docking domain consists of negatively charged acidic residues (common docking domain (CD)) suggesting this site of interaction relies



Type I = MKP-6, VHR	Type III = MKP-5
Type II = MKP-1. MKP-2, MKP-3, MKP-4, MKP-X, PAC1, VH3	Type IV = VH5, MKP-7

Figure 11 – 1.10.2.1 Subtypes of DSPs

Cartoon illustrating the domain differences between members of the mammalian DSP family. Members belonging to each subtype are indicated (in box). *NT* refers to an H terminal extension; *MKB* refers to MAP kinase-binding; *DSP* refers to dual specificity phosphatase catalytic domain; *PEST* refers to degradation sequences as well as a C terminal extension. Conserved residues present in both the MKB and DSP domains are also shown.

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on electrostatic forces. All DS-MKPs contain the extended active site signature motif HCXXXXXR common to all protein phosphatases which retains a high sequence homology between family members (37-50%) (fig. 11). The crystal structures of phosphatases MKP-3 and VHR reveal these phosphatases contain a shallow cleft active site which can accommodate both the phosphotyrosine and phosphothreonine side chains of activated MAPK (Stewart et al., 1999; Yuvaniyama et al., 1996). This and other work suggests MKP-3 undergoes rearrangements upon substrate binding, therefore bringing together the residues required for catalytic activation (Camps et al., 1998; Farooq et al., 2001). Recent data suggests this may be applicable to other phosphatases including MKP-2 (Chen et al., 2001a), MKP-4 (Camps et al., 1998) and MKP-1 (Hutter et al., 2000). In the latter case, the cause of this conformational change could be through phosphorylation, as the binding of MKP-1 to ERK1 results in the phosphorylation of MKP-1. This does not result in the direct activation of MKP-1 but in the stabilisation of the protein by the reduction in its ability to be degraded (Brondello et al., 1999; Sohaskey and Ferrell, 2002). Therefore, this may exist as a mechanism of negative feedback potentially as a universal way of regulating MAPK. To continue, once in an active state, cysteine and arginine residues along with aspartate exist together in the active site of these phosphatases. These residues are critical to substrate MAPK dephosphorylation. The mechanism utilised by these DSPs to dephosphorylate their substrates has been described through characterisation of MKP-3 inactivation of ERK2 (Zhao and Zhang, 2001). These data suggest dephosphorylation of pThr and pTyr by MKPs occurs in a stage-dependent manner, where DSPs preferentially dephosphorylate

pTyr in order to initiate the reaction. They suggest the thiolate anion of cysteine initially targets the phosphoryl group of phospho-tyrosine in the p-Thr-pTyr-MAPK for nucleophilic attack. At the same time, the aspartate donates a hydrogen anion to the phenolic oxygen of the MAPK tyrosine, resulting in the release of pThr-MAPK and formation of pMKP-intermediate. The aspartate group accepts a proton from a water molecule and the left over hydroxyl group attacks the remaining cysteinyl-phosphate residue. This results in the release of orthophosphate (Pi) and return of the thiolate anion in the active site of the MKP. Another MKP molecule of the same type is then thought to dephosphorylate the remaining pThr residue of the MAPK. The arginine and histidine residues in the signature motif of MKPs are thought to stabilise the phosphoryl groups during catalysis and maintain the thiolate anion for nucleophilic attack, respectively (Denu and Dixon, 1998).

A third domain exists in JNK specific MKPs VH5/M3/6 and MKP-7 which consists of a unique C terminal extension (fig. 11). It has been suggested residues within this region control the stability and localisation of these MKP members including PEST sequences (Rechsteiner and Rogers, 1996) as well as nuclear import and export sequences (Masuda et al., 2001; Tanoue et al., 2001b). Along with PEST sequences, residues 463-511 and 569-604 of the C terminus have been shown , when modified, to target MKP-7 for degradation through ubiquitination (Katagiri et al., 2005). However, as an alternative mechanism of cross talk and regulation between MAPK pathways, ERK2 has been shown to phosphorylate Ser-446 present in the C terminal extension of MKP-7 under particular growth signals (Masuda et al., 2003). In doing this, ERK2 stabilises MKP-7 which increases its half life leading to an

enhanced inhibition of JNK activation (Katagiri et al., 2005). This phosphorylation has also been seen in the MKP-1/ERK relationship as seen above suggesting it may be a common mechanism.

Work performed to analyse the regulation of these DSPs indicates some of these phosphatases including MKP-1/CL100 (Lewis et al., 1995; Liu et al., 1995; Sun et al., 1993) , B23/VH3 (Ishibashi et al., 1994; Kwak and Dixon, 1995), PAC1/VHR (Rohan et al., 1993), M3/6/VH5 (Johnson et al., 2000; Theodosiou et al., 1996) and MKP-3 (Groom et al., 1996) can be induced by different factors and stresses, whereas MKP-5 (Tanoue et al., 1999) and MKP-7 (Tanoue et al., 2001b) are not rapidly inducible. PAC1 can be induced by p53, via transcriptional regulation, to negatively regulate the ERK cascade in p53-induced apoptosis and growth suppression in response to serum deprivation and oxidative stress (Yin et al., 2003). Under phorbol myristate acetate and v-ras dependent stimulation of haemopoietic cells, PAC1 transcription can be blocked by the introduction of a dominant negative ERK2 mutant (Grumont et al., 1996). This suggests PAC1 induction is mediated via MAPK activation, potentially as a feedback mechanism similar to other situations described above. It has also been demonstrated that under stimulation by growth factors the Raf/MEK/ERK pathway can induce and regulate MKP-1 expression (Cook et al., 1997). More detailed analysis shows MKP-1 has the ability to activate both Raf-1 and MEK1 & -2 as a mechanism of positive feedback (Shapiro and Ahn, 1998).

DSPs potentially regulate MAPKs which control growth, development and differentiation; these data have also suggested DSPs can theoretically act as tumour

suppressor proteins. Chromosomal analysis has revealed that the locations of many DSPs correspond to sites known to have oncogenic potential (Denkert et al., 2002; Montpetit et al., 2002; Muda et al., 1997; Theodosiou et al., 1996; Wang et al., 2003).

Despite the wealth of information known about these MKPs and their ability to inactivate specific MAPKs, there is little direct *in vivo* evidence confirming this relationship in mammals. Some of the clearest evidence for a physiological role for MKPs comes from analysis of MKP-3 in the developing chick embryo. MKP-3 has a patterned expression in places throughout the developing mouse embryo associated with fibroblast growth factor (FGF) signaling (Dickinson et al., 2002). Follow up studies show MKP-3 expression is regulated via the FGF-ERK pathway during early development and therefore exists as part of a negative feedback loop. This is a direct role for MKP-3 in regulating a MAPK signaling pathway that plays a key role in development (Eblaghie et al., 2003; Smith et al., 2005). However, genetic disruption studies in a mammalian system of MKP-1, shows no obvious phenotypic abnormality or disruption to regulation of MAPKs (Dorfman et al., 1996). This lack of phenotype could exist due to redundancy between family members. Mice deficient in MKP-5 have recently been characterised and show no developmental abnormalities. This study does suggest a role for MKP-5 in the immune response. This is discussed further below.

As described above, the phosphatase interaction with substrate MAPK is as essential for retaining specificity of phosphatase function as MAPKK interactions are with MAPK. It is therefore important to understand the relationships of these phosphatases and their substrates. Proposed DSP substrate MAPK specificities are

indicated in figure 10. Biochemical studies have shown different regions of the phosphatases are required for binding different substrate MAPKs (Masuda et al., 2003; Slack et al., 2001; Tanoue et al., 2002). However, whether these phosphatases have one or more substrates *in vivo* is still unclear. The lack of understanding is partially due to problems with the over expression assays used and their inability to reflect the *in vivo* response and true substrate specificity. Therefore these MKP targets should be treated with caution as they may be specific to cell type, physiological status or the promiscuous nature of phosphate exchange.

1.10.2.2 DS-MKPs which preferentially dephosphorylate JNK

There are 3 members of this family that mainly target JNK for dephosphorylation; MKP-5, VH5 (M3/6) and MKP-7.

1.10.2.2.1 MKP-5

Originally cloned in 1999 this protein binds and specifically dephosphorylates the stress activated MAPKs, p38 and JNK (Tanoue et al., 1999; Theodosiou et al., 1999). It also has an extremely low affinity *in vitro* towards the ERK group of MAPKs.

As well as typical structural motifs representative of all DSPs including a Cdc25-like domain and C terminal phosphatase motif, MKP-5 contains an extended N terminal or catalytic domain (fig. 11). In a similar manner to the MKPs with extended C terminal domains, this region is considered to play a role in protein-protein interactions, potentially controlling substrate specificity or the stability of

MKP-5. MKP-5 mRNA is present at high levels in the liver and skeletal muscle tissues (Tanoue et al., 1999), which is a restricted distribution in comparison to other DSPs, suggesting a more defined function. MKP-5 is evenly distributed between the cytoplasm and nucleus, which remains unchanged after stimulation with different stresses. p38 and JNK localise to the cytoplasm and nucleus in resting and stressed cells, respectively. It is therefore possible MKP-5 can regulate p38 and JNK present in these locations. Although MKP-5 is not an inducible member of the DSP family, elevated mRNA levels are detected through treatment with TNFα, anisomycin and osmotic shock but not UV. However, new data suggest MKP-5 expression can be induced in macrophages by stimulation with LPS and can be down regulated in activated T cells (Zhang et al., 2004). The chromosomal location of MKP-5 corresponds to a region which has been associated with renal collecting duct carcinoma (CDC) (Steiner et al., 1996) and some breast cancers (Benitez et al., 1997). One of the few genetic studies attempting to describe the physiological role of DSPs *in vivo* was shown using a MKP-5-deficient mouse. MKP-5 null mice develop normally, however analysis of the T cells identifies enhanced activity of JNK but surprisingly, not p38. In addition, MKP-5 -/- antigen presenting cells have an enhanced ability to activate antigen specific T cells when compared to wild type cells suggesting MKP-5 negatively regulates this function in APCs. However, MKP-5 seems to also play a role in T cell proliferation, as MKP-5 -/- activated T cells proliferate poorly. This suggests MKP-5 positively regulates this characteristic of T cells and therefore all together plays more than one role in regulating the immune

response. Hence, this study definitively identifies a physiological function for MKP-5 and therefore, one of the first roles of MKPs in mammals.

1.10.2.2.2 VH5/M3/6

M3/6 was cloned in the mouse (Theodosiou et al., 1996) and is 90% homologous to the human homolog VH5 (Martell et al., 1995) at the amino acid level. M3/6 preferentially targets JNK, and to a lesser extent p38, for inactivation but not ERK (Muda et al., 1996). JNK is able to phosphorylate M3/6 on one of 12 phosphorylation sites present in the C terminal region of M3/6 (Johnson et al., 2000) potentially to stabilise the protein. M3/6 contains a motif similar to the c-Jun/JNK interaction site (Adler et al., 1992), which when mutated prevents phosphorylation of M3/6 by JNK and blocks M3/6-mediated inactivation of JNK (Johnson et al., 2000). This suggests an interaction is required between JNK and M3/6 in order for JNK inactivation to occur. It is a large protein that when first described was the first DSP to locate to the cytoplasm but has subsequently also been isolated in the nucleus (Theodosiou et al., 1996). Sequence analysis identified this protein as having an extended active site similar to other DSPs, the CH2 motifs and interestingly a complex trinucleotide repeat which is unique to M3/6 and resides within the extended C terminal domain also common to MKP-7. M3/6 has an expression pattern limited mainly to the brain. The location of the M3/6 gene on mouse chromosome 7 is consistent with the human VH5 gene location on chromosome 11. This region on chromosome 11 is responsible for some non-small cell lung carcinomas, indicating VH5 and therefore M3/6 may act as tumour

suppressor proteins (Bepler and Garcia-Blanco, 1994). This coincides with the function of JNK in regulating proliferation and differentiation and that dysregulation of JNK can lead to the formation of some tumours as described earlier in the chapter. It has been suggested, M3/6 is down regulated or inactivated after exposure to protein damaging stresses including osmotic & heat shock and hydrogen peroxide, in order to allow sustained JNK activation to occur (Chen et al., 2001b; Palacios et al., 2001).

1.10.2.2.3 MKP-7

Cloned in 2001, MKP-7 added to the group of DSPs which preferentially inactivate JNK and p38 (Masuda et al., 2001; Tanoue et al., 2001b). Expression of gain of function constitutively active JNK isoforms α and β, caused the induction of MKP-7 in COS-7 cells (Han et al., 2002). This suggests MKP-7 is induced in response to JNK hyperactivation, suggesting that MKP-7 is a negative regulator of JNK. In both primary sequence and structural domains MKP-7 is most similar to M3/6. It is expressed at low levels in many tissues but highly in the brain, heart and testes. Interestingly, this phosphatase contains a similar C terminal stretch contained by its closest family member M3/6. As described earlier, this region contains sites for ERK phosphorylation which when modified help stabilise the protein, suggesting MKP-7 action on JNK could be regulated through a ERK feedback mechanism. Although located mainly to the cytoplasm, MKP-7 contains both nuclear import and export sequences within this C terminal region which allow it to enter the nucleus (Tanoue et al., 2001b). These sequences may enable MKP-7 to translocate to

the nucleus to dephosphorylate target substrates and then transport them back to the cytoplasm, functioning thus as a shuttle protein (Masuda et al., 2001). The sequences within these extended C terminal domains of M3/6 and MKP-7 are so similar it is possible M3/6 is also able to act as a nuclear shuttle protein. As described earlier, the C terminal extension of MKP-7 also contains PEST sequences (high in proline, glutamate, serine and threonine residues). PEST sequences have been frequently found in proteins which are rapidly degraded suggesting they target these proteins for proteolysis. They also contain 2 stretches of high sequence similarity (373-406 & 596-665 amino acids), which show no sequence homology to other proteins. These regions may hold sites for interactions with proteins specific to these larger MKPs. It has been suggested both the N terminal stretch in MKP-5 and the C terminal stretches in M3/6 and MKP-7 could act to regulate intrinsic phosphatase activity through binding to other proteins or formation of an inhibitory conformation (Tanoue et al., 1999) as shown with other DSP members (Hutter et al., 2002). An MKP highly homologous to MKP-7, also able to inactivate JNK has recently been reported, namely MKP-M (Matsuguchi et al., 2001). This phosphatase is involved in the desensitisation of LPS-mediated JNK activation and cytokine secretion in macrophages (Matsuguchi et al., 2001; Musikacharoen et al., 2003). MKP-7 has been mapped to a region of chromosome 12 responsible for many tumours associated with leukaemia conferring a function of MKP-7, similar to other members, as a tumour suppressor protein (Hoornaert et al., 2003; Montpetit et al., 2002).

1.11 Aims of this study

The aim of this work was to attempt to understand how JNK can be negatively regulated by dual specificity phosphatases. As described above, there is evidence to suggest JNK and specific upstream kinases associate with the scaffold proteins, JIP-1 and -2 (Whitmarsh et al., 1998; Whitmarsh et al., 2001) and that the formation of these modules enhances stress induced JNK activation. The idea that these scaffold proteins bind these modules and localise them to distinct subcellular regions to specifically regulate JNK, suggested the potential possibility scaffold proteins could also aid the regulation of JNK by binding JNK specific negative regulators.

The work presented here identifies a functional interaction between JIP-1 and DSPs, MKP-7 and M3/6. This along with other work was published in the Journal of Biological Chemistry (Willoughby et al., 2003). In addition, this thesis also presents work which identifies a dynamic functional interaction between the GPCR adaptor protein and JNK3 scaffold, β-arrestin 2, and MKP-7 which has also been published in the Journal of Biological Chemistry (Willoughby and Collins, 2005).

Chapter 2: Materials and Methods

2.1 Plasmids and molecular biology

pcDNA3-T7-JIP-1, -2, -3, pEBG-JIP-1, pcDNA3-Flag-JNK2α2, pcDNA3-MKK7, pcDNA3-HA-JNK3, pcDNA3-HA-ASK1, pcDNA3-GFP-β-arrestin 2 and pcDNA3-Flag-β-arrestin 2 were provided by Dr Alan Whitmarsh. The pcDNA3-HA-AT1aR construct was a gift from Dr Stephane LaPorte. pcDNA3.1-MKP-7, Xp- M3/6, pcDNA4hisC-MKP-4 and pcDNA4hisC-MKP-7 C-S were provided by Dr Gordon Perkins. Expressed sequence tags (ESTs) were obtained for MKP-1(h), MKP-2(h), MKP-7(h), PAC1(h), MKP-3(h), and β-arrestin 2(h), (IMAGE) consortium identification numbers: 4794895, 3605895, 4400399, 4297852, 5183133 and 3028154. All phosphatases were subcloned into pcDNA4hisC (Invitrogen) under the control of a CMV promoter and fused to an N terminal Xpress™ tag epitope. β-arrestin 2 and all β-arrestin 2 deletions and JIP-3 were subcloned into the pEBG vector. pEBG is a glutathione S-transferase (GST) fusion vector derived from the insertion of a PCR generated BglII-BamH1 GST fragment from pGEX-2T (Pharmacia) into the BamH1 site of pEBB (Sanchez et al., 1994). pEBB was derived from the pEF-BOS expression vector containing the gene promoter for human elongation factor 1α (Mizushima and Nagata, 1990). All sequences were validated through sequencing.

2.1.1 Oligonucleotides used in the polymerase chain reaction (PCR)

Construct Name	Forward Primer (5-3)	Reverse Primer (5-3)	Restriction Sites
pCDNA4his C-T7/Xp-MKP-7	CGGGATCCACCATGGCC CATGAGATGATTG	CCGCTCGAGTCAGGAG ACCTCAATGATTTC	BamH1 & Xho1
pCDNA4his C-T7/Xp-MKP-2	CGGGATCCACCATGGT GACGATGGAGGAG	ATAGTTTAGCGGCCGC TAACAGCTGGGAGAGT	BamH1 & Not1
pCDNA4his C-T7/Xp-hPAC1	GGGGTACCGATGGGG CTGGAGGCGGCG	GGAATTCTCAGTGAC ACAGCACCTG	Kpn1 & EcoR1
pCDNA4his C-T7/Xp-MKP-3	CGGGATCCACCATGATA GATACGCTCAGACCCG	CCGCTCGAGTCACGTA GATTGCAGAGAGTCC	BamH1 & Xho1
pCDNA4his C-T7/Xp-MKP-1	CGGGATCCACCATGG TCATGGAACTGGGC	GGAATTCTCAGCAG CTGGGAGAGGT	BamH1& EcoR1
pCDNA4his C-T7/Xp-MKP-5	CGGGATCCACCATGCC TCCGTCTCCTTTAG	GCGAATTCACACAA CCGTCTCCAC	BamH1& EcoR1
pCDNA4his C-T7/Xp-MKP-7 Δ360	CGGGATCCACCATGGC CCATGAGATGATTG	CCGCTCGAGCTAGCT GGCGGGATGCACGGG	BamH1 & Xho1
pCDNA4his C-T7/Xp-MKP-7 Δ394	CGGGATCCACCATGGC CCATGAGATGATTG	CCGCTCGAGCTAATT GCTGTCTTCCAGCCTG	BamH1 & Xho1
pCDNA4his C-T7/Xp-MKP-7 Δ443	CGGGATCCACCATGGC CCATGAGATGATTG	CCGCTCGAGCTAGCA TAGCTTGTTGGTCCC	BamH1 & Xho1
pCDNA4his C-T7/Xp-MKP-7 Δ522	CGGGATCCACCATGGC CCATGAGATGATTG	CCGCTCGAGCTAGGAA AGGCCGAAAAGGAAG	BamH1 & Xho1
pCDNA4his C-T7/Xp-MKP-7 Δ552	CGGGATCCACCATGGC CCATGAGATGATTG	CCGCTCGAGCTAGG AAGGGGTAGAGGTCT G	BamH1 & Xho1
pCDNA4his C-T7/Xp-MKP-7 C term	CGGGATCCGTGCCC AGCGTGCCCAGC	GGATCCTCAGGAGA CCTCAATGAT	BamH1 & Xho1
GST-JIP-3	ACTAGTATGATGGAG ATCCAGATG	TATTCTTGCGGCCGCT C ACTCAGGGGTGTAGGA	Spe1 & Not1
pCDNA4his C-T7/Xp-JNK3	GAAGATCTACCATG GGTCGGGATCTGTAC	CCGCTCGAGTCACC TGCAACAACCCAG	BglII & Xho1
GST-β-arrestin 2	CGGGATCCACCATG GGGGAGAA	TATTCTTAGCGGCCGC C	BamH1 & Not1

		TAGCAGAGTTGATCAT	
GST-βarr2 1-164	CGGGATCCACCATG GGGGAGAA	TAGCGGCCGCCTAAGA GTTCCTTTTGTGGCTTT TC	BamH1 & Not1
GST-βarr2 165-410	CGGGATCCACCATGGTG CGGCTGGTGATCCGAAA G	TATTCTTAGCGGCCGC C TAGCAGAGTTGATCAT C	BamH1 & Not1
GST-βarr2 165-195	CGGGATCCACCATGGTG CGGCTGGTGATCCGAAA G	TAGCGGCCGCCTAGTC AGACATGAGGAAGTG GCGT	BamH1 & Not1
GST-βarr2 165-239	CGGGATCCACCATGGTG CGGCTGGTGATCCGAAA G	TAGCGGCCGCCTAGTA CTGTCTCACAGAGACT TTG	BamH1 & Not1
GST-βarr2 165-278	CGGGATCCACCATGGTG CGGCTGGTGATCCGAAA G	TAGCGGCCGCCTACAG TGGGGTTATGGTGTAC ACC	BamH1 & Not1
GST-βarr2 165-363	CGGGATCCACCATGGTG CGGCTGGTGATCCGAAA G	TAGCGGCCGCCTACTC GGCAGCGGCTGACTGG GGT	BamH1 & Not1
GST-βarr2 189-410	CGGGATCCACCATGCGC CACTTCCTCATGTCTGAC	TATTCTTAGCGGCCGC C TAGCAGAGTTGATCAT C	BamH1 & Not1
GST-βarr2 202-410	CGGGATCCACCATGGCT TCCCTGGACAAGGAGCT G	TATTCTTAGCGGCCGC C TAGCAGAGTTGATCAT C	BamH1 & Not1

2.1.2 Preparation of heat shock competent *E. coli*

1ml of an overnight culture of *E. coli* HB101 or DH5α growing in LB (Invitrogen) was expanded into 100ml of fresh LB and incubated with shaking for 2h at 37°C. The culture was put on ice for 10 minutes, and then pelleted at 4°C and the supernatant discarded. The bacterial pellet was resuspended in 30ml ice-cold transformation buffer (10mM Pipes, 55mM MnCl, 15mM CaCl, 250mM KCl). Then the bacteria were pelleted and subsequently resuspended in 10ml ice-cold transformation buffer and then frozen at -80°C in aliquots.

2.1.3 Transformation of bacteria

100µl of competent bacteria were mixed with 10µl of ligation reaction or appropriate concentration of plasmid DNA and incubated on ice for 15 minutes. The mixture was then incubated at 42°C for 80 seconds, and placed back on ice for at least 2 minutes. Bacteria were then plated onto LB-agar plates containing 50µg/ml ampicillin (Sigma) and incubated overnight at 37°C.

2.1.4 Preparation of plasmid DNA 'miniprep' and 'midiprep'

A single bacterial colony grown overnight on a LB-agar plate containing ampicillin was picked and grown overnight at 37°C with shaking in either 5ml (miniprep) or 100ml of LB medium containing 50µg/ml ampicillin. The following day, bacteria were pelleted by centrifugation at 5000 *g* for either 10 minutes (miniprep) or 25 minutes (midiprep). With the supernatant removed, plasmid DNA was extracted using the Marligen mini prep kit (Bomi UK) or Qiagen plasmid midi kit in both circumstances following the manufacturer's instructions.

2.1.5 Digestion of DNA using restriction enzymes

Both plasmid DNA and PCR products were cut with 10U of specific restriction enzyme(s) (Promega) and mixed with the appropriate 1X Buffer indicated by the manufacturer (Promega). Digests were incubated at 37°C either overnight (with the addition of mineral oil) or between 1-3 hours depending on the manufacturer's instructions.

2.1.6 Analysis of DNA using agarose gel electrophoresis

Plasmid DNA, PCR products and DNA products from restriction digests were separated by electrophoresis on 1% agarose gels (SeaKem GTG agarose, Cambrex) containing 0.5μg/ml ethidium bromide (Sigma). The gel was cast and run in 1X TAE buffer (0.4M Tris-acetate, 1mM EDTA). 0.75μg of 1Kb DNA ladder was run alongside the samples as a size marker (Invitrogen). If required, DNA fragments were excised from the gel.

2.1.7 Purification of DNA from agarose gel

DNA fragments were excised from agarose gel and purified using the CONCERT™ Rapid Gel Extraction System (Invitrogen) according to the manufacturer's instructions.

2.1.8 Ligation of DNA fragments

50-100ng of cut plasmid DNA was incubated with three times the concentration of insert DNA (from restriction digest or PCR) and added to Solution I of the TaKaRa DNA Ligation Kit Ver.2.1 which contains T4 DNA Ligase. The sample was then incubated at either 16°C for 1h or overnight at 4°C. The whole ligation mixture was then used to transform competent *E. coli.*

2.1.9 Amplification of DNA sequences using PCR

Where PCR was used to create DNA fragments, template DNA (either EST or plasmid DNA) (~1ng) was added to 1X *Ex Taq*™ Buffer containing 2mM MgCl, as well as 2.5mM of each dNTP (dATP, dCTP, dGTP, dTTP), 1μM of each oligonucleotide primer and 5U of *Ex Taq*™ (TaKaRa, Japan) to a final volume of 50μl. In some circumstances the final volume included the addition of either 4% DMSO (Sigma) or 38% Betaine (Sigma). Samples were then overlaid with mineral oil (Sigma) and the PCR reaction performed in a Hybaid Thermocycler. Under standard conditions, typical cycles consisted of:

1 cycle of 96°C for 1 minute

30-35 cycles of 96°C for 1 minute + primer Tm for 2 minutes + 72°C for 3 minutes

1 cycle of 75°C for 5 minutes

An aliquot of the resulting DNA fragments was visualised on agarose gels and if correct the rest of the sample was purified as described below. Cleaned PCR fragments where indicated were then digested with the appropriate restriction enzyme(s) and ligated into the desired plasmid vector.

2.1.10 Purification of plasmid DNA and PCR fragments

An equal volume of TE Buffer was added to both PCR amplified DNA fragments or plasmid DNA, and to this the same volume of Buffer-saturated Phenol (Invitrogen) was added. After strong vortexing, the sample was spun for 5 minutes and the top layer of the resulting sample removed to a new sterile eppendorf. A tenth of this volume of NaAc was added back to the sample, as well as 2.5 x the

volume as 99% ethanol and the sample vortexed. The DNA was then incubated at -20°C for 30 minutes and then pelleted for 15 minutes. All the supernatant was removed and the pellet washed with 500µl 70% ethanol, and supernatant removed and pellet air dried on ice. The sample was then dissolved in 10µl of HPLC distilled water.

2.2 Cell culture

2.2.1 Cell lines

The 293T cell line is a human embryonic kidney derived cell which expresses the SV40 large T antigen. The COS-7 cell line from African Green Monkey kidney was originally derived from simian CV-1 cells transformed with SV40 antigen. Both cell lines are adherent.

2.2.2 Culture conditions

The adherent cell lines were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (FCS) and 50µg/ml Streptomycin, 50µg/ml Penicillin in a 10% CO_2 humidified incubator at 37°C. To passage, cells were washed with Hanks' Balanced Salt Solution (HBSS) (Invitrogen) and incubated with Trypsin (Invitrogen) at 37°C for 5 minutes. For long term storage, cells were trypsinised, washed with HBSS and then resuspended in freeze mix (10% DMSO, 90% FCS), stored at -20°C for 6 hours, then moved to -80°C overnight and finally to liquid nitrogen until required.

2.2.3 Transfection of cells with plasmid DNA

Transfer of plasmid DNA was carried out using a liposomal transfection reagent, LipofectAMINE™ (Invitrogen) using the protocol described by the manufacturer's.

2.2.4 Inhibition of nuclear export by Leptomycin B

293T cells transfected with pCDNA4hisC-tagged MKP-7 (0.5μg) were grown sparsely on glass cover slips and starved overnight with serum-free medium. The cells were then washed once with HBSS and incubated with 40nM Leptomycin B (Sigma) for 0, 30, 60, 120 and 180 minutes. The cover slips were then prepared for immunofluorescent analysis as described below.

2.2.5 Incubation of cells with Angiotensin II

Cells either seeded on glass cover slips or 5 x 10^7 cells in $6cm^2$ wells were transfected with the required DNA including the Angiotensin Type 1a Receptor (AT1aR). The cells were left for 48 hours, washed with HBSS and then incubated with 1μM Angiotensin II (Sigma) for either 0, 5, 15, 30 or 60 minutes. Stimulated cells were then either prepared for immunofluorescent analysis or extracts made and used for protein analysis, both as described below.

2.3 Protein analysis

All experiments were performed at least 3 times.

2.3.1 Preparation of cell lysates

Cells were washed once with ice-cold Phosphate Buffered Saline (PBS) and whole cell extracts of both 293T and COS-7 cells were made for phosphorylation studies through direct lysis into 200µl gel loading buffer (50mM Tris pH 6.8, 100mM Dithiothreitol (Sigma), 2% SDS (BDH), 4% Glycerol (BDH), 0.4% Bromophenol Blue (Sigma)). 293T cells were lysed in 500µl of ice-cold Tris-HCL lysis buffer for GST Pull down or immunoprecipitation experiments (50mM Tris pH 7.5, 150mM NaCl, 5mM EDTA (BDH), 1% IGEPAL (Sigma), 4% proteinase inhibitor cocktail tablets (Roche)).

2.3.2 GST Pull down and immunoprecipitation

For pull down or immunoprecipitation, samples were incubated on ice for 10 minutes and then centrifuged at 14,000 *g* for 10 minutes at 4°C. Supernatants were recovered and subjected to pull down or immunoprecipitation. Cell extracts were then either incubated with glutathione-sepharose 4B (Amersham Biosciences) for GST-containing constructs, or with protein G-Sepharose beads (Sigma) along with the appropriate antibody (either anti-T7 tag antibody or M2 anti-Flag tag antibody) for 3 hours at 4°C. Beads were then washed 3 times in ice-cold lysis buffer and resuspended in an appropriate volume of gel loading buffer (final concentration 50mM Tris pH6.8, 2% SDS, 100mM dithiothreitol, 4% glycerol).

2.3.3 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples containing protein extract in gel loading buffer as described above were heated at 90°C for 5 minutes. SDS-PAGE was performed using lower separating gel made with 375mM Tris pH 8.8, 0.1% SDS and 10% acrylamide (BIO-RAD 30% Acrylamide/Bis solution, 37.5:1) and upper stacking gel made with 125mM Tris pH6.8, 0.1% SDS and 5% acrylamide both containing 0.1% Temed (Sigma) and 0.1% ammonium persulphate (Sigma). The separating gel solution was poured between clean glass plates (Cambridge Electrophoresis) and overlaid with 500µl Tris pH 8.8 saturated Butanol and left to polymerise. After polymerisation the gel was washed with HPLC-purified water to remove excess butanol and the stacking gel layered over the separating gel and allowed to polymerise. Benchmark Prestained Ladder (Invitrogen) size markers were run in every gel alongside protein samples with equivalent concentrations. Gels were run at between 150-200V in running buffer (25mM Tris base (Sigma), 200mM Glycine (BDH) and 0.1% SDS). Proteins were then transferred to nitrocellulose membranes.

2.3.4 Electrotransfer of separated proteins

For immunoblotting separated proteins were transferred to nitrocellulose membrane (Hybond™-ECL™ Amersham Biosciences) by semi-dry electrotransfer (Hoefer) using Transfer buffer (50mM Tris-base, 380mM glycine, 0.1% SDS) containing 20% methanol at 15 V for 40 minutes. Efficiency of transfer was checked by staining the membrane with Ponceau S (Sigma).

2.3.5 Immunoblotting

Membranes were blocked in blocking solution I (PBS, 0.1% Tween-20 (Sigma) and 5% milk powder (Tesco)) with gentle rocking overnight at 4°C. They were then subjected to immunoblotting with primary antibody (shown in 2.3.6) in blocking solution II (PBS, 0.1% Tween-20 and 1% milk powder) with gentle rocking for either 1-4 hours at room temperature or overnight at 4°C. The membrane was then washed 3 times for 10 minutes with an excess volume of PBS-T (0.1% Tween-20). The presence of bound primary antibody was then detected on the membrane using the appropriate antibody conjugated to horseradish peroxidase (HRPO) (shown in 2.3.6) in blocking solution I for 1 hour with gentle rocking. Membranes were then washed 3 times for 10 minutes in excess volume of PBS-T. They were developed using enhanced chemiluminescence (ECL™ Amersham Biosciences) as described by the manufacturer's instructions. They were briefly dried on blotting paper and covered in plastic wrap and then exposed to X-ray film (Hyperfilm ECL Amersham Biosciences) for the appropriate time (between 5-120 seconds).

2.3.6 Antibodies

Antibody Name	Species	Concentration	Manufacturer
Anti-T7 tag monoclonal	Mouse	1/1000	Novagen
Anti-MKP-7 polyclonal	Rat	1/3000	(Willoughby et al., 2003)
Anti-Xpress tag monoclonal	Mouse	1/5000	Invitrogen
Anti-M3/6/GST polyclonal	Rabbit	1/1000	(Palacios et al., 2001)
Anti-Flag M2 tag monoclonal	Mouse	1/2000	Sigma
Anti-Myc tag monoclonal	Mouse	1/2000	(Evan et al., 1985)
Anti-active JNK antibody	Rabbit	1/2000	Promega
Anti-HA tag monoclonal	Rat	1/2000	Roche
Anti-Mouse HRPO-conjugated IgG	Rabbit	1/2000	Dako
Anti-Rat HRPO-conjugated IgG	Rabbit	1/2000	Dako
Anti-Rabbit HRPO-conjugated IgG	Pig	1/2000	Dako

2.3.7 Monoclonal anti-MKP-7 antiserum

In order to improve our ability to detect both over expressed and endogenous MKP-7 we attempted to make an anti-MKP-7 rat monoclonal antibody. Rats were immunised with a complete MKP-7 peptide via Peyer's patches, then lymph node

cells were fused with the rat myeloma line, Y3-Ag 1.2.3 as described (McKnight et al., 1996). Hybridomas were expanded and supernatants were screened for antibodies to MKP-7 by enzyme-linked immunosorbent assaying (ELISA). We further identified positive clones through western blot analysis (figure 12, left panel). However, after purification of selected positive clones and by various purification methods, we failed to improve the quality of this antibody in detecting endogenous MKP-7, in fact it ended up being less specific than our previous rat polyclonal (figure 12, right panel).

2.3.8 Densitometry

Densitometry software GeneSnap (SynGene) was used to assign arbitrary units to represent the phosphorylation of JNK3 by ASK1 or under activation of the AT1aR. These units were then presented in a graphical format to allow clear comparison of JNK3 phosphorylation under different conditions.

2.4 Confocal microscopy

2.4.1 Immunofluorescence analysis

After transfection and stimulation with either leptomycin B or angiotensin II, cells grown on cover slips were washed gently with phosphate-buffered saline (PBS) solution. They were then fixed at room temperature with 4% paraformaldehyde (Sigma) for 15 minutes and permeabilised with 0.2% Triton X-100/PBS (Sigma) for 15 minutes at room temperature. Indirect immunofluorescence was performed by

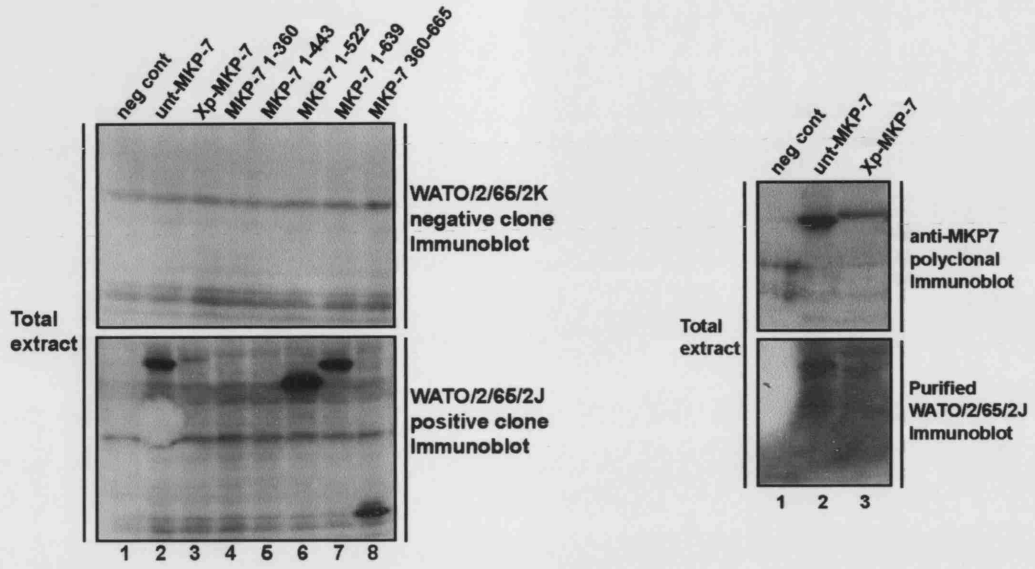

Figure 12 – 2.3.7 Western blot to show specificity of MKP-7 monoclonal

Left panel – 293T cells were either left untransfected or transfected with untagged MKP-7 (0.75μg), Xp-tagged MKP-7 (0.75μg) or Xp-tagged MKP-7 deletions (0.75μg) (see figure 16b). The following day 5 x 10^6 cells were lysed directly into gel loading buffer and the samples separated by SDS-PAGE. After electrotransfer to nitrocellulose membrane, monoclonal supernatants from MKP-7 immunized rats were used in immunoblotting to test their ability to detect MKP-7. Negative and positive examples of the supernatants are shown (WATO/2/65/2K and WATO/2/65/2J, respectively).

Right panel - 293T cells were either left untransfected or transfected with untagged MKP-7 (0.75μg) or Xp-tagged MKP-7 (0.75μg). The next day 5 x 10^6 cells were lysed directly into gel loading buffer and the samples then separated by SDS-PAGE. After electrotransfer to nitrocellulose membrane, either the previously described anti-MKP-7 rat polyclonal or purified rat monoclonal, WATO/2/65/2J, were used in an immunoblot to detect MKP-7.

incubation with the MKP-7 polyclonal antiserum in 1% bovine serum albumin/0.5% goat serum albumin/PBS for 1 hour at 37°C. The cells were then washed 3 times for 5 minutes at room temperature and then incubated with Texas-Red-conjugated secondary antibody (Jackson Immunoresearch) for 1 hour at 37°C. Cells were then thoroughly washed again 3 times for 5 minutes at room temperature, the cover slips lightly dried and mounted on glass slides using mounting medium (Vectashield, Vector Laboratories Inc.). The images were prepared on a Zeiss confocal microscope using BioRad Lasersharp software.

Chapter 3: The JIP family of scaffold proteins

3.1 Introduction

As described in chapter 1, the JIP family of proteins can act as scaffold proteins for the JNK group of MAPKs (Kelkar et al., 2000; Lee et al., 2002; Whitmarsh et al., 1998; Yasuda et al., 1999). By binding upstream kinases these proteins can act to enhance JNK and corresponding substrate, c-Jun, activation. Based on sequence similarity the JIP proteins are split into two groups. JIP-1 and JIP-2 are structurally very similar, with both having SH3 and PTB domains which bind many proteins. JIP-3 and JIP-4 are structurally different from JIP-1 and JIP-2 containing extended coiled-coil and leucine zipper domains, although they can also function as MAPK scaffolds. Apart from MAPKs, MLKs, MKK7 and numerous JNK isoforms and in the case of JIP-4 p38, these proteins can interact with other signaling proteins as described earlier, including the light chain of the kinesin motor protein, RhoGEF, amyloid precursor protein, HPK and some members of the LDL receptor family including ApoER2. Through these interactions with kinesin and other signaling molecules, the JIP proteins can transport signaling modules to specific subcellular sites, including the cell periphery, as a way to regulate JNK signaling.

As detailed in chapter 1, JNK activity is regulated by protein phosphatases. This is essential for the maintenance of JNK activity at its basal level in resting cells and after stimulation. DSPs can specifically target MAPKs for dephosphorylation. The DSP family of 10 main members can be separated into sub-groups through classification of their structure, tissue distribution and MAPK specificity. Three

MKPs preferentially inactivate JNK; MKP-5, M3/6 and MKP-7. MKP-1 is able to inactivate JNK, p38 and ERK to the same degree.

The work presented in this chapter attempts to identify whether DSPs known to dephosphorylate JNK can utilise the JIP scaffold proteins in order to regulate JNK.

3.2 Results

3.2.1 Selective Binding of JIP-1 and JIP-2 to the DSP MKP-7

JIP-1, -2 and -3 bind MAPK signaling components as described in chapter 1 (Kelkar et al., 2000; Whitmarsh et al., 1998; Yasuda et al., 1999). MKP-7 is one DSP which preferentially targets and regulates JNK (Masuda et al., 2001; Tanoue et al., 2001). I examined whether this DSP could associate with the JIP family of scaffold proteins to specifically regulate JNK activity. I co-expressed epitope-tagged JIP-1, JIP-2 and JIP-3 with MKP-7 and after immunoprecipitation examined the JIP precipitates for the presence of MKP-7 using an MKP-7 polyclonal rat antibody (Willoughby et al., 2003). JIP-1 and JIP-2 but not JIP-3 associated with MKP-7 (fig. 13, compare lanes 6, 9 and 12). This suggests MKP-7 can interact with specific subsets of JNK scaffold proteins.

3.2.2 JNK specific DSP M3/6 can also bind JIP-1, whereas MKP-5 and other DSP family members cannot

M3/6 and MKP-5 are also members of the JNK specific DSP sub-group (Muda et al., 1996; Tanoue et al., 1999; Theodosiou et al., 1999). To assess whether these JNK phosphatases can also bind to the JIP proteins, Gordon Perkins in our laboratory co-expressed epitope tagged M3/6 along with GST-JIP-1 to assess the interaction (data not shown). He showed M3/6 can bind both JIP-1 and JIP-2 but not JIP-3 (Willoughby et al). I wanted to assess whether MKP-5 could bind to the JIP proteins. I co-expressed MKP-5 along with GST-JIP-1 and assessed the precipitates

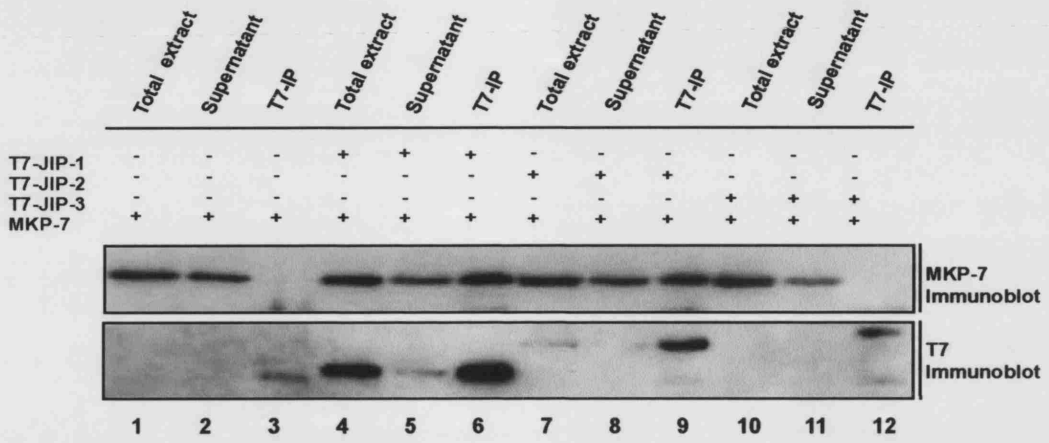

Figure 13 – 3.2.1 Selective binding of JIP-1 and JIP-2 to the DSP MKP-7

Constructs expressing T7-tagged JIP-1, JIP-2, JIP-3 and pCDNA3 (control lanes 1-3) (0.2μg) were transfected into 293T cells along with a plasmid expressing MKP-7 (0.75μg). Cells were left overnight, extracts were made of 5 x 10^6 cells and JIP-containing complexes were isolated by immunoprecipitation using an anti-T7 tag antibody. These precipitates were separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane examined for the presence of MKP-7 by immunoblot using a polyclonal MKP-7-specific antiserum. The presence of JIP proteins and MKP-7 in the total extract and supernatant are also shown.

for the presence of MKP-5 using an anti-Xpress tag antibody. MKP-5 does not bind to JIP-1 either (fig. 14, lane 2). A control lane shows an interaction between MKP-7 and JIP-1 as a positive control (fig. 14, lane 4). These data suggest distinct members of the JNK specific sub-group of DSPs can interact with the JIP family of proteins.

To then examine whether the JIP proteins selectively interacted with JNK specific DSPs MKP-7 and M3/6, I co-expressed GST-tagged JIP-1 and synthetically tagged DSPs MKP-2, MKP-4 and PAC1. GST Pull down analysis demonstrated that MKP-7 detected by immunoblot was present in the JIP-1 precipitates (fig. 15, lane 8). In contrast I saw no interaction with the DSPs MKP-2, MKP-4 or PAC1 (fig. 15, lanes 2, 4 and 6). Our laboratory has also shown MKP-1 is unable to bind JIP-1 (data not shown). These data demonstrate that JIP-1 binds to a restricted subset of DSPs including MKP-7 and M3/6.

3.2.3 MKP-7 and M3/6 bind to JIP-1 independently of JNK binding

MKP-7 (Masuda et al., 2001; Tanoue et al., 2001) and M3/6 (Muda et al., 1996) are able to bind JNK. JIP-1 also binds directly to JNK (Whitmarsh et al., 1998; Yasuda et al., 1999). The formation of complexes between JIP-1 and MKP-7 or M3/6 therefore might be through a common interaction with JNK. Gordon Perkins performed JIP-1 deletion analysis and showed that MKP-7 can still bind to a JIP-1 mutant that lacks the JNK Binding Domain (Willoughby et al). These data indicated that the C terminal region of JIP-1 is required for phosphatase binding and that the phosphatases can form stable interactions with JIP-1 in the absence of

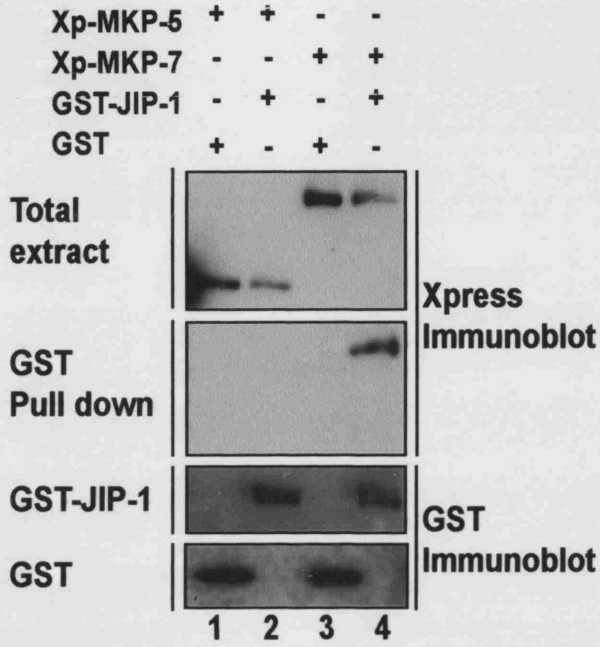

Figure 14 – 3.2.2 DSP MKP-5 cannot bind JIP-1

Constructs expressing GST or GST-JIP-1 (0.2μg) were introduced into 293T cells along with Xp-tagged MKP-5 or MKP-7 (0.75μg). Extracts were made the next day from 5 x 10^6 cells and GST-JIP-1-containing precipitates were isolated using glutathione-sepharose beads (*GST Pull down*). These precipitates were then separated by SDS-PAGE and following electrotransfer to nitrocellulose membrane the presence of either MKP-5 or MKP-7 was identified by immunoblot using the anti-Xpress tag antibody. The presence of all constructs is also shown in the *total extracts.*

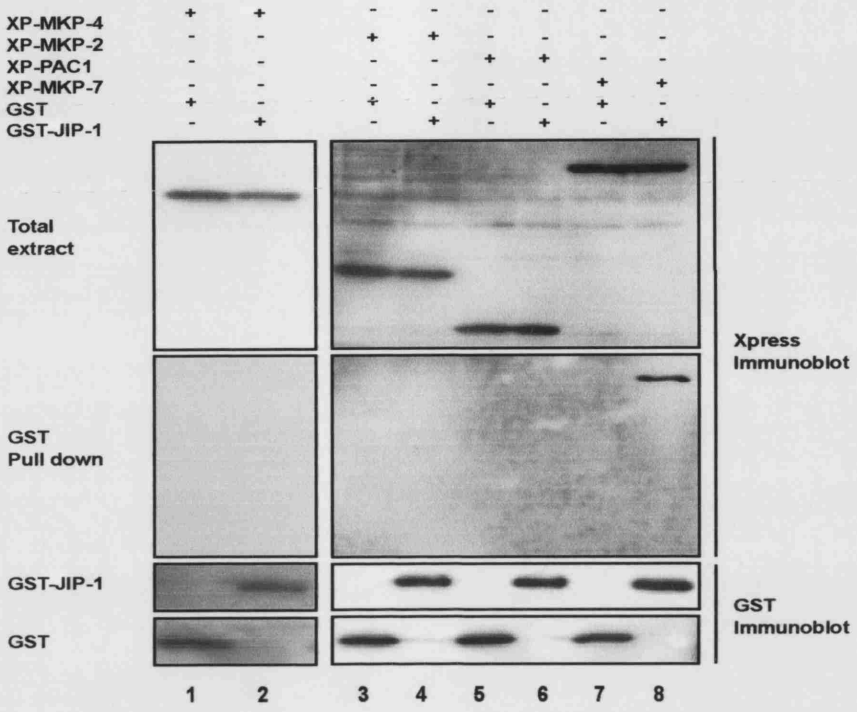

Figure 15 – 3.2.2 MKP-4, MKP-2 and PAC1 do not bind JIP-1

Plasmids containing GST or GST-JIP-1 (0.2μg) were expressed with either Xp-tagged MKP-2, MKP-4, hPAC1 or MKP-7 (0.75μg) in 293T cells. Cells were left overnight, extracts were made of 5 x 10^6 cells and GST-JIP-1-containing complexes were isolated using glutathione-sepharose beads (*GST Pull down*). These precipitates were separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of the phosphatases was examined by immunoblot using the anti-Xpress tag antibody. The presence of GST, GST-JIP-1 and Xp-tagged phosphatases are also shown in the *total extracts*.

JNK. This, however, does not rule out the possibility that JNK helps to stabilise the interaction between JIP-1 and the phosphatases. I next sought to identify the regions within MKP-7 and M3/6 which mediate binding to JIP-1. I performed a GST Pull down from cells expressing MKP-7 deletion mutants (fig. 16a) and GST-JIP-1 (fig. 16b). A MKP-7 deletion mutant containing amino acids 1-394 did not bind to JIP-1, whereas a mutant containing amino acids 1-443 did bind JIP-1, albeit more weakly than full-length MKP-7 (fig. 16b, compare lanes 1, 5 and 7). These experiments identify the sequence between amino acids 394 and 443 as important for binding. There was increased binding to JIP-1 of a MKP-7 mutant containing amino acids 1-552 (fig. 16b, lane 11), indicating that additional sequences within the C terminus also may contribute to binding. Furthermore, the C terminal fragment of MKP-7 (residues 360-665) was sufficient for binding to JIP-1 (fig. 25, compare lanes 2 and 8) whereas residues 1-394 did not bind (fig.25, lane 5). The C terminal region of MKP-7 that binds JIP-1 is distinct from the JNK binding domain located in the N terminal region of MKP-7 (Tanoue et al., 2001). As shown in fig. 17, the MKP-7 1-394 construct, which cannot bind JIP-1, can still bind JNK2α2 (fig.17, lane 3). Thus MKP-7 binding to JIP-1 is dependent on residues within its C terminal region. This region is sufficient for the interaction therefore demonstrating the interaction is independent of JNK binding to MKP-7. Work performed by our collaborator Alan Whitmarsh showed this interaction leads to the specific dephosphorylation of the JIP-1 bound subset of JNK and subsequent decrease in activation of c-Jun (Willoughby et al., 2003).

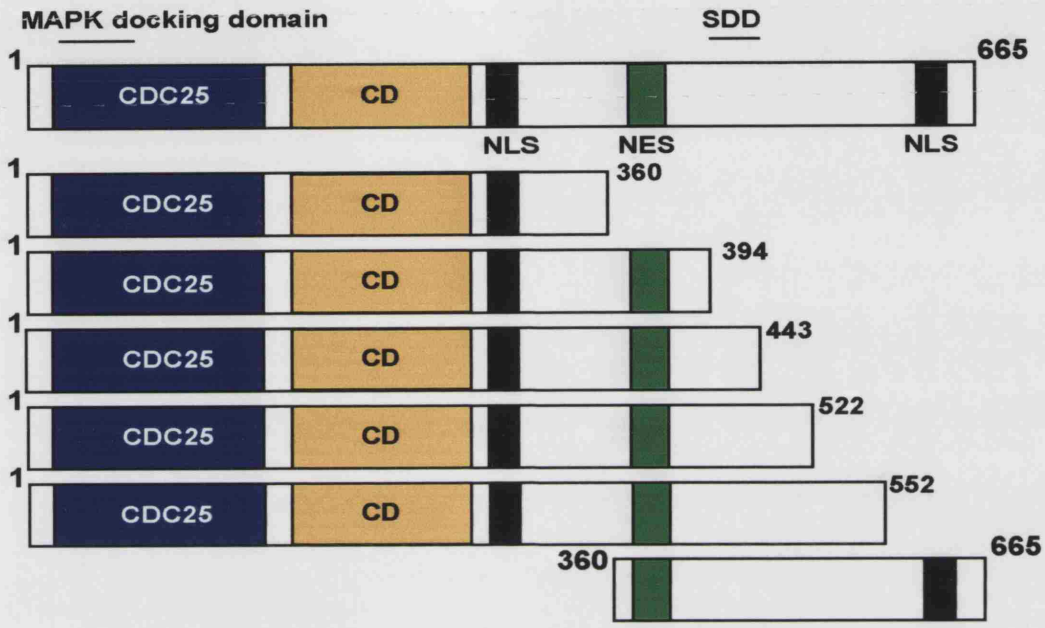

Figure 16(a) – 3.2.3 MKP-7 deletion constructs

Cartoon representing MKP-7 deletion constructs made to assess the interaction between scaffolds JIP-1 and β-arrestin 2 with MKP-7. *Numbers* refer to the amino acid position. *SDD*, Proposed scaffold docking domain identified in these experiments; *CDC25*, CD25 homology domain, *CD*, DSP catalytic domain; *NLS*, nuclear localisation sequence; *NES*, nuclear export sequence.

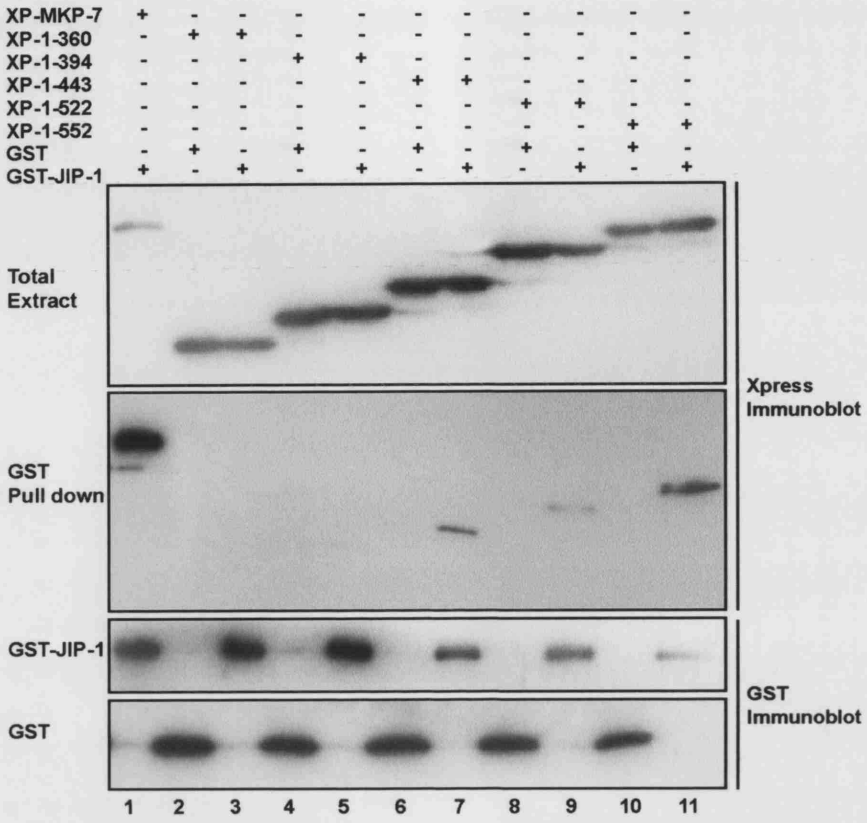

Figure 16(b) – 3.2.3 MKP-7 binds to JIP-1 through residues 394-443

Plasmids containing GST or GST-JIP-1 (0.2μg) were introduced along with Xp-tagged MKP-7 or MKP-7 deletion constructs 1-360, 1-394, 1-443, 1-522 and 1-552 (0.75μg) to 293T cells. The cells were left overnight and extracts were made the following day from 5 x 10^6 cells. GST-containing complexes were isolated from the extract using glutathione-sepharose beads (*GST Pull down*) and then separated by SDS-PAGE. After electrotransfer to nitrocellulose membrane, the presence of MKP-7 or MKP-7 deletions was examined by immunoblot using the anti-Xpress tag antibody. The presence of GST, GST-JIP-1, MKP-7 and deletion constructs are shown in the *total extracts.*

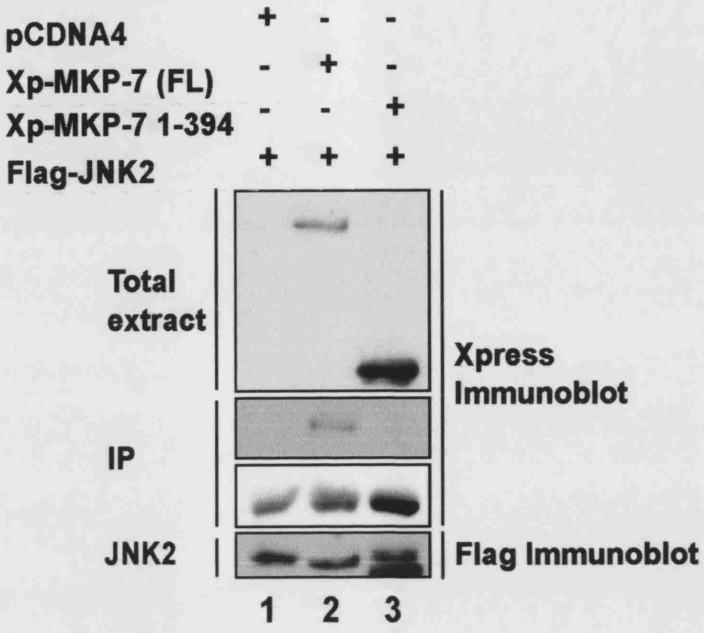

Figure 17 – 3.2.3 MKP-7 binds JIP-1 through a region independent of JNK

Plasmids expressing Flag-JNK2 (0.5μg) were expressed in 293T cells along with Xp-tagged MKP-7 or MKP-7 1-394 (0.75μg). The following day extracts were made from 5 x 10^6 cells and JNK2-containing complexes were isolated by immunoprecipitation using the anti-Flag M2 monoclonal antibody. These complexes were separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of MKP-7 and MKP-7 1-394 was examined by immunoblot using the anti-Xpress tag antibody. The presence of all the plasmids are also shown in the *total extracts*. Notice the heavy chain of the antibody is present in all lanes however, in the presence of MKP-7 1-394 the band is much heavier indicating the presence of the phosphatase in the immunoprecipitated complex.

The extended C terminus of MKP-7 is homologous to that of M3/6 but no other DSP which possibly explains the selective binding of MKP-7 and M3/6 to JIP-1. I examined the region of M3/6, which mediates binding to JIP-1 and identified the C terminus of M3/6 as important for binding (data not shown). Gordon Perkins performed experiments suggesting M3/6 binds JIP-1 independently of JNK and that this interaction allows the specific dephosphorylation of JIP-1 bound JNK by M3/6 (data not shown).

3.2.4 MKP-3 binds to JIP-3

JIP-3/JSAP1 has been shown to regulate JNK during LPS stimulation of Toll-like receptor 4 (Matsuguchi et al., 2003) as well as in response to hydrogen peroxide stimulation (Matsuura et al., 2002). JIP-3 is the neuronal isoform of the JIP family but has low homology with members JIP-1 and -2. As discussed above, JIP-3 does not bind M3/6 or MKP-7. I therefore performed GST Pull down analysis which showed DSP MKP-3 strongly interacts with JIP-3 (fig. 18, lane 4). PAC1 also bound to JIP3 albeit a lot weaker than MKP-3 (fig. 19, lane 4). These preliminary data suggest specific JIP proteins may recruit specific DSPs to regulate JNK in different cellular environments or subcellular localisations. It would be interesting to perform further experiments to identify a system where JIP-3 utilises MKP-3 to regulate a bound component. A good candidate for this system would be the TLR4 signaling system.

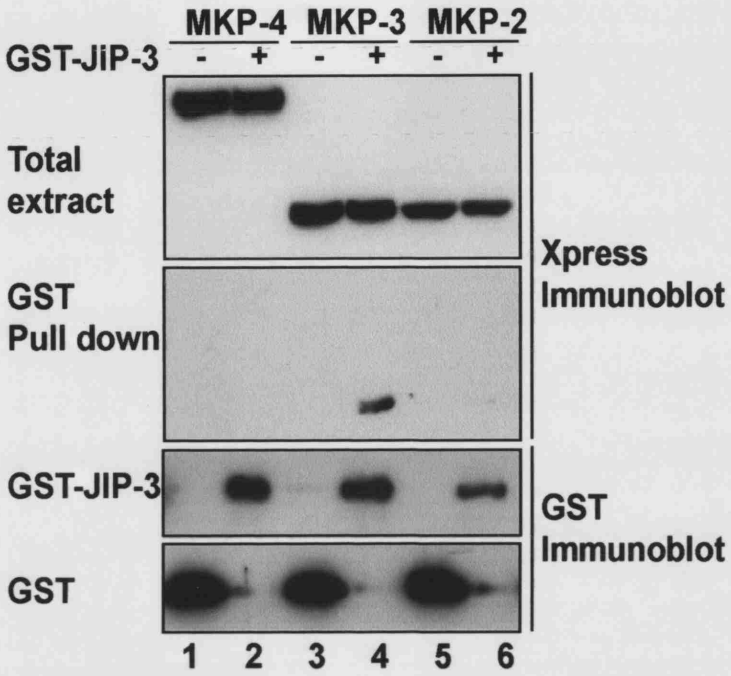

Figure 18 – 3.2.4 MKP-3 and MKP-2 bind JIP-3

Constructs containing GST or GST-JIP-3 (0.2μg) were expressed along with either Xp-tagged MKP-2, MKP-3 or MKP-4 (0.75μg) in 293T cells. The cells were left overnight and extracts then made from 5 x 10^6 cells. JIP-3-containing precipitates were obtained using glutathione-sepharose beads (*GST Pull down*) and these complexes were then separated by SDS-PAGE. After electrotransfer to nitrocellulose membrane, the presence of the phosphatases was examined by immunoblot using the anti-Xpress tag antibody. The presence of all the plasmids are also shown in the *total extracts*.

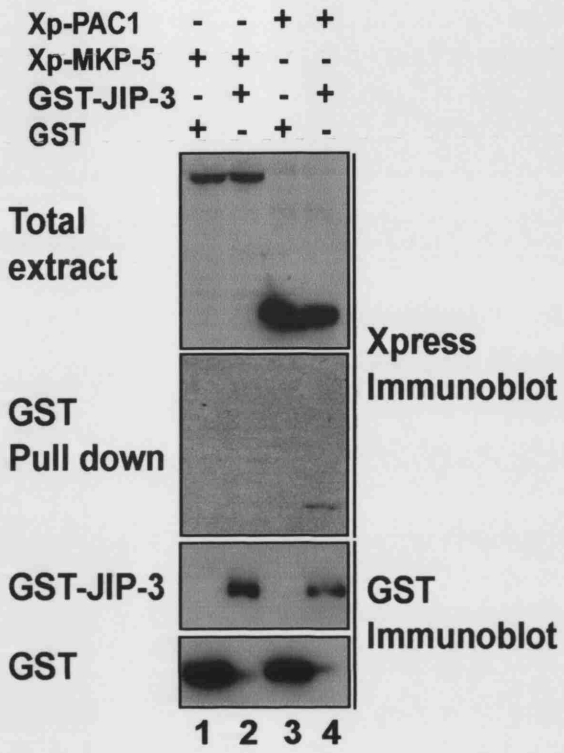

Figure 19 – 3.2.4 PAC1 binds JIP-3

Constructs containing GST or GST-JIP-3 (0.2μg) were expressed in 293T cells along with either Xp-MKP-5 or Xp-PAC1 (0.75μg). Extracts were made the following day from 5 x 10^6 cells and GST-JIP-3 containing complexes were isolated using glutathione-sepharose beads (*GST Pull down*). These complexes were separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of the phosphatases examined by immunoblot using the anti-Xpress tag antibody. The relative protein levels of all plasmids are also shown in the *total extracts*.

3.2.5 MKP-7 1-360 is an intact and catalytically active protein

To assess whether the MKP-7 C terminus (residues 360-665) which binds JIP could control its ability to regulate JNK, I co-expressed MKK7, JNK and either FL-MKP-7 or MKP-7 residues 1-360 and assessed the extracts for the presence of phosphorylated JNK (fig. 20). Preliminary data suggested that by removing the C term of MKP-7, its ability to dephosphorylate JNK was enhanced (fig. 20, compare lanes 5 and 6, 7 and 8, 9 and 10, 11 and 12). This shows that this deletion construct is still functional and able to dephosphorylate JNK, which is important to know in the experiments which use the truncated forms of MKP-7.

Initially I considered this enhanced activity to be the result of a loss of regulatory residues within the MKP-7 C term. However, recently published data has shown that it is more likely the loss of PEST and other critical sequences present within the MKP-7 C term which could cause the supposed increase in MKP-7 'activity'. These residues cause MKP-7 to be ubiquitinated, after which the phosphatase is targeted for proteosomal degradation (Katagiri et al., 2005). Therefore, by removing the C term along with these residues, MKP-7 becomes more stable and this increased stability may be what is seen here (fig. 20). However, comparison of the protein loads of FL MKP-7 and MKP-7 C term shows no difference suggesting these proteins are not being degraded at different rates (fig. 20, compare lanes 3 and 4, 5and 6, 7 and 8, 9 and 10).

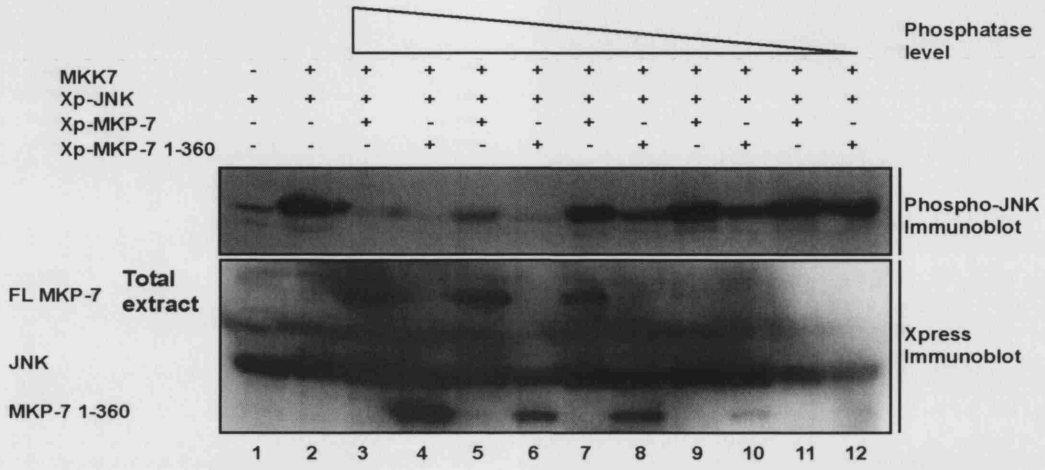

Figure 20 – 3.2.5 MKP-7 is an intact and catalytically active protein

Constructs expressing MKK7 (0.1μg), Xp-tagged JNK (0.325μg) were introduced into 293T cells along with titrated levels of Xp-tagged MKP-7 or Xp-tagged MKP-7 1-360 (0.3μg, 0.1μg, 0.03μg, 0.01μg, 0.004μg). Cells were left overnight, and total extract were then made from 5 x10^6 cells. The whole cell extract was separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane examined for the presence of phospho-JNK by immunoblot using an antibody raised against the phosphorylated form of JNK. The presence of total JNK and MKP-7 constructs are also shown.

3.3 Discussion

These data help provide the first evidence that specific dual specificity phosphatases can be recruited onto MAPK scaffold proteins. Some of this work was published along with additional experiments, showing that the scaffold proteins JIP-1 and JIP-2 recruit MKP-7 and M3/6 in order to specifically inactivate JNK and consequentially reduce the activation of the JNK substrate, c-Jun (Willoughby et al., 2003). The specific residues required for the interaction between JIP-1 and MKP-7 will be discussed later.

These data also suggest JIP-3 can recruit specific phosphatases including MKP-3 and PAC1. Within this assay, MKP-3 was able to bind JIP-3 more than PAC1. Interestingly, all three DSPs preferentially target ERK as their substrate, although also have the ability to dephosphorylate JNK. Over expression analysis showed JIP-3 binds JNK1, JNK2 and most strongly JNK3 but did not bind ERK2 (Kelkar et al., 2000). Kuboki et al present data suggesting JIP-3 can bind ERK signaling components, Raf-1 and MEK1, in order to suppress ERK activation (Kuboki et al., 2000). It is possible that these experiments may not have been sensitive enough to detect the presence of ERK2 or another ERK isoform may. Alternatively, JIP-3 may bind ERK-specific DSPs to target them to their lesser preferred substrate JNK or to further suppress ERK activation. The latter reason would suggest distinct scaffold proteins could specify target substrates for phosphatases potentially controlled by their subcellular localisation or under specific stimuli.

It is clear the JIP family of proteins are able to scaffold regulating components of the JNK pathway. When and how these components enter and work within the scaffold system has yet to be elucidated. Through the accumulation of JIP proteins at specific subcellular sites, the local concentration of MAPK components is increased. Under these conditions, both JIP-1 and JIP-2 form homo- and hetero-oligomeric complexes and it is thought this may be a mechanism of ensuring specific activation of the required signaling components. Within the active signaling module, it is unclear whether the phosphatases are already bound or whether they are recruited to the scaffold by a particular stimuli or specific subcellular location. However, immunofluorescence analysis of both scaffold and phosphatase subcellular locations, suggests the proteins both co-localise and have distinct patterns of staining (Willoughby et al., 2003). Indicating a specific subset of each protein interacts at one time and therefore these phosphatases may be recruited to the scaffold under specific conditions.

The ability of scaffolds to recruit proteins to regulate MAPK activity is not unique to DSPs. As suggested in chapter 1, Akt can also bind to JIP-1 and in doing so inhibit the interaction of JIP-1 with JNK and therefore inhibit JNK activity. Under kainate exposure, Akt binding to JIP-1 is reduced and JNK binding to JIP-1 is thus increased allowing activation of JNK (Kim et al., 2002). In addition, JNK recruitment to the JIP-1 scaffold has the ability to reduce the binding between JIP-1 and MLK as a way of regulating its own activation by the MAP2K (Nihalani et al., 2001).

The physiological role of the JIP proteins and therefore the function of the JNK pathways signaling through these scaffolds including DSPs are only now being uncovered. JIP plays an essential role in stress-induced neuronal cell death associated with JNK (Whitmarsh et al., 2001), cytokine induced cell death in pancreatic cells (Haefliger et al., 2003) and Alzheimer's disease through the interaction with the amyloid precursor-protein (APP) (Scheinfeld et al., 2003). More recently JIP was shown to regulate JNK and its effect on insulin resistance in obesity (Jaeschke et al., 2004). JIP-2 has been shown to interact with the Reelin receptor ApoER2 and JIP-3 with Toll-like receptor 4 (TLR4). These last instances have identified signaling systems under which the phosphatases identified from this work could regulate the bound MAPK. Other JNK scaffolds which can also regulate receptor signaling include β-arrestin 2 (McDonald et al., 2000).

The work here has identified a novel interaction between a MAPK regulator and DSP, however situations where scaffold proteins have utilised both positive and negative regulators have been seen in other signaling systems including the family of A-kinase anchoring proteins (AKAPs) (Westphal et al., 1999). Along with the work presented here, this may suggest a common mechanism of scaffold-bound MAPK regulation.

Chapter 4: Dynamic interaction between MKP-7 and β-arrestin 2

4.1 Introduction

The work presented in chapter 3 identified a functional interaction between the JNK scaffold protein, JIP and members of the DSP family, specifically MKP-7 and M3/6. This interaction leads to the specific dephosphorylation of the JIP-bound subset of JNK (Willoughby et al., 2003) and subsequent decrease in c-Jun dephosphorylation. Analysis of the dynamics between these proteins is limited through lack of reagents and an easily testable, well studied physiological role for the JIP protein. To further my analysis of these scaffold binding phosphatases, I attempted to identify an alternative JNK scaffold protein which had a well characterised physiological function.

Alternative JNK scaffolds include β-arrestin 2, CRK, filamin and POSH; their characterisation was discussed in chapter 1. β-arrestin 2 is an adaptor protein critical for the desensitisation and internalisation of G protein coupled receptors via clathrin coated pits. In order to achieve this, β-arrestin 2 can bind to a plethora of proteins essential for receptor signaling internalisation including clathrin, AP-2, NSF, Ral-GDS, ERK and ARNO. The binding sites for these proteins have not been fully elucidated. As suggested above, as well as this classical role, β-arrestin 2 is able to scaffold specific components of the JNK signaling pathway, JNK3, ASK1 and indirectly MKK4 (McDonald et al., 2000). In doing so β-arrestin specifically enhances JNK3 activation through over expression of ASK1 and activation of the GPCR, angiotensin type 1a receptor (AT1aR). These were the first experiments to

identify a scaffold protein which brings a JNK module under the control of a receptor signaling system. This function of β-arrestin 2 suggested it to be a good candidate for the analysis of DSP scaffold bound JNK regulation.

This chapter attempts to identify a link between DSPs and JNK scaffold β-arrestin 2, in order to further understand the relationship between JNK scaffold proteins and DSPs.

4.2 Results

4.2.1 MKP-7 binds β-arrestin 2

β-arrestin 2 has been identified as a scaffold protein for JNK3, the neuronal isoform of JNK (McDonald et al., 2000; Miller et al., 2001). As shown in chapter 3, DSPs MKP-7 and M3/6 bind the JNK scaffold proteins JIP-1 and JIP-2 in order to specifically inactivate JNK (Willoughby et al., 2003). I wanted to assess whether M3/6 and MKP-7 could also bind β-arrestin 2. I co-expressed GST-tagged β-arrestin 2 with Xpress-tagged M3/6 and MKP-7 and examined β-arrestin 2 precipitates for presence of the phosphatases using the anti-Xpress tag antibody. Under these conditions, MKP-7 bound β-arrestin 2 (fig. 21 top panel, lane 2), whereas the highly related M3/6 was not detected (fig. 21, bottom panel, lane 2). Data from our laboratory has shown, M3/6 binds JIP-1 much less than MKP-7 (data not shown). Here MKP-7 binds β-arrestin 2 much more weakly than JIP-1 therefore it may be possible this assay is not sensitive enough to detect the presence of M3/6.

To assess whether other DSP family members could bind to β-arrestin 2, I performed GST pull down experiments on cells expressing GST-β-arrestin 2 and Xpress tagged phosphatases. MKP-1, MKP-2, PAC1, MKP-4 and MKP-5 were not detected in the β-arrestin 2 precipitates (fig. 22, lanes 2, 4 & 6, and fig. 23, lanes 4 and 6).

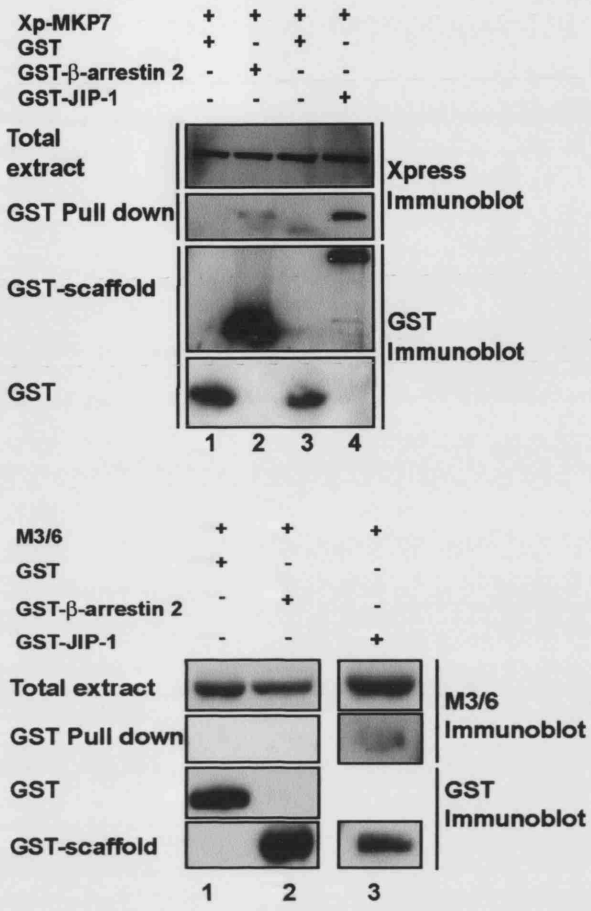

Figure 21 – 4.2.1 MKP-7 but not M3/6 binds beta-arrestin 2

Top panel – GST, GST-β-arrestin 2 and GST-JIP-1 (0.2μg) were expressed in 293T cells along with MKP-7 (0.75μg). The cells were left overnight and then GST-containing complexes were isolated using glutathione-sepharose beads (*GST Pull down*) from 5 x 10^6 cells. These complexes were separated using SDS-PAGE and electrotransferred to nitrocellulose membrane. The presence of MKP-7 was in the GST precipitates examined by immunoblot using the anti-Xpress tag antibody. The expression levels of all proteins used is also shown in the *total extracts.*

Bottom panel - GST, GST-β-arrestin 2 and GST-JIP-1 (0.2μg) were expressed in 293T cells along with DSP M3/6 (0.75μg). The following day GST complexes were isolated using glutathione-sepharose beads (*GST Pull down*) from 5 x 10^6 cells. Samples were separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of M3/6 in the GST precipitates examined by immunoblot using a polyclonal antiserum for M3/6. The expression levels of GST, GST-β-arrestin 2, GST-JIP-1 and M3/6 are also shown in the *total extracts.*

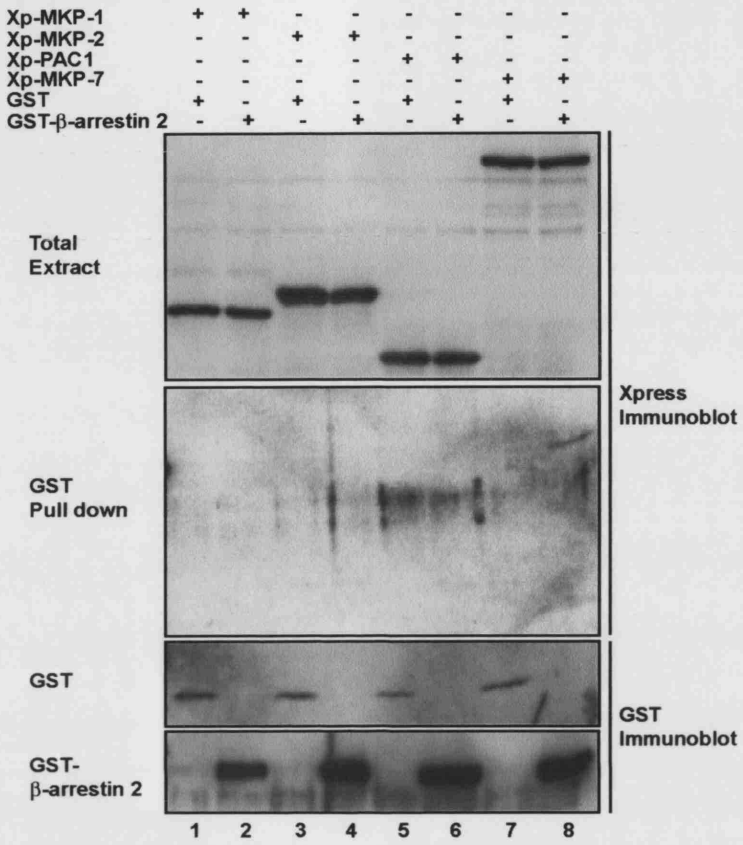

Figure 22 – 4.2.1 MKP-1, MKP-2 and PAC1 do not bind beta-arrestin 2

Plasmids expressing Xp-tagged MKP-1, MKP-2, PAC1 and MKP-7 (0.75μg) were introduced into 293T cells together with GST and GST-tagged β-arrestin 2 (0.2μg). β-arrestin 2 containing complexes were isolated the following day using glutathione-sepharose beads (*GST pull down*) from 5 x 10^6 cells. The precipitates were separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of the phosphatases in the β-arrestin 2 precipitates examined by immunoblot using the anti-Xpress tag antibody. The relative expression levels of GST, GST-β-arrestin 2 and the DSPs are also shown in the *total extracts*.

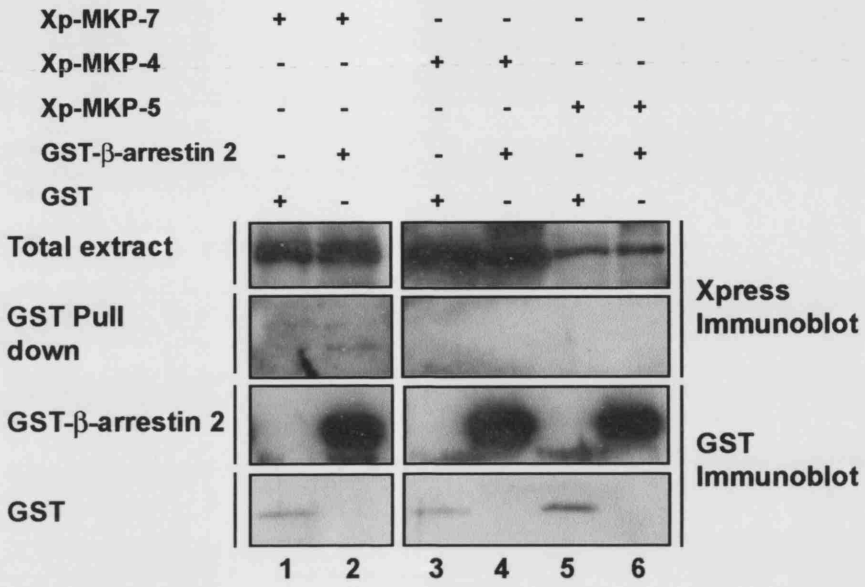

Figure 23 – 4.2.1 MKP-4 and MKP-5 do not bind beta-arrestin 2

Plasmids expressing GST and GST-β-arrestin 2 (0.2μg) were introduced into 293T cells together with Xp-tagged MKP-7, MKP-4 or MKP-5 (0.75μg). The next day extracts were made from 5 x 10^6 cells and GST-containing complexes were obtained using glutathione-sepharose beads (*GST Pull down*). These precipitates were separated using SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of phosphatase examined by immunoblot using the anti-Xpress tag antibody. The relative expression levels of all proteins are also shown in the *total extracts.*

4.2.2 MKP-7 binds to β-arrestin 2 through the same region as JIP-1

As shown in chapter 3, MKP-7 uses specific residues within its C terminal fragment to bind JIP-1 (Willoughby et al., 2003) (fig. 16b). I wanted to assess whether these C terminal residues also confer binding to β-arrestin 2. GST pull down analysis of GST-β-arrestin 2 expressed along with MKP-7 deletion mutants (Fig. 16a) identified a deletion mutant containing amino acids 1-394 that did not bind β-arrestin 2, whereas a mutant containing amino acids 1-443 did (fig. 24, Top panel, lanes 6 and 8). The sequence between amino acids 394 and 443 is also critical for MKP-7 binding to JIP1. The equivalent region of M3/6 is very similar to that of MKP-7, which suggests either that β-arrestin 2 unlike JIP1 discriminates between the two sequences, or that M3/6 binding is not detected within the sensitivity of our assay as previously suggested. Furthermore, the MKP-7 C terminal fragment (residues 360-665) was sufficient for binding both JIP-1 and β-arrestin 2, whereas residues 1-394 did not bind (fig. 25, compare lanes 5, 6, 8 and 9). As with JIP-1, binding to β-arrestin 2 still occurs with a fragment which is unable to bind JNK (fig. 17). This suggests MKP-7 to β-arrestin 2 binding is not through JNK and the C terminal residues of MKP-7 are sufficient for this interaction.

4.2.3 MKP-7 interacts with the central region of β-arrestin 2 independently of JNK

To discover the binding site for MKP-7 on β-arrestin 2, I performed GST Pull down analysis of β-arrestin 2 deletion constructs (fig. 26) expressed together with MKP-7.

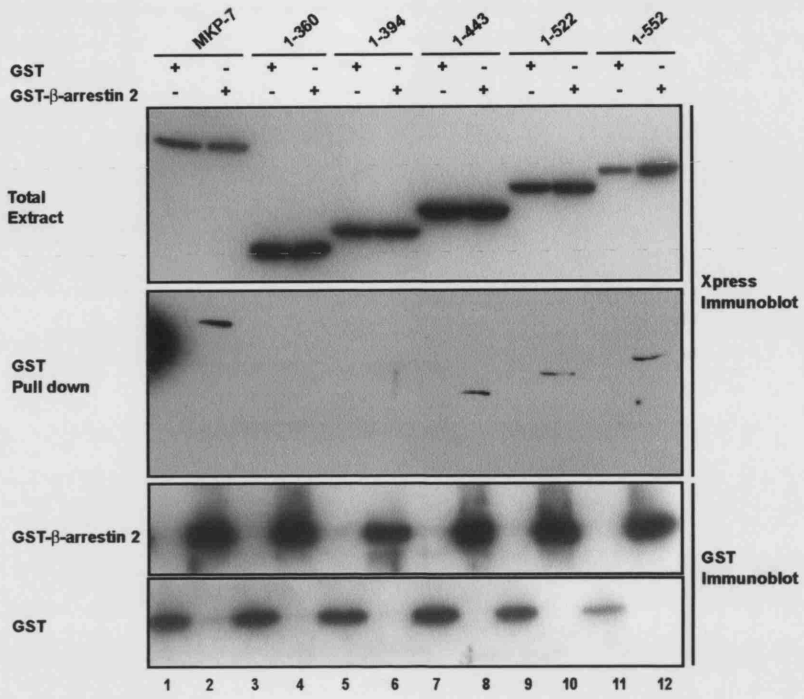

Figure 24 – 4.2.2 MKP-7 uses the residues 394-443 to bind beta-arrestin 2

Constructs expressing GST or GST-β-arrestin 2 (0.2μg) were introduced into 293T cells together with XP-tagged MKP-7 deletion constructs shown in figure 16a (0.75μg). Extracts were made the following day from 5 x 10^6 cells and β-arrestin 2 containing complexes were isolated using glutathione-sepharose beads (*GST Pull down*). These precipitates were separated using SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of MKP-7 in the GST precipitates was examined by immunoblot using the anti-Xpress tag antibody. Relative expression levels of all proteins are also shown in the *total extracts.*

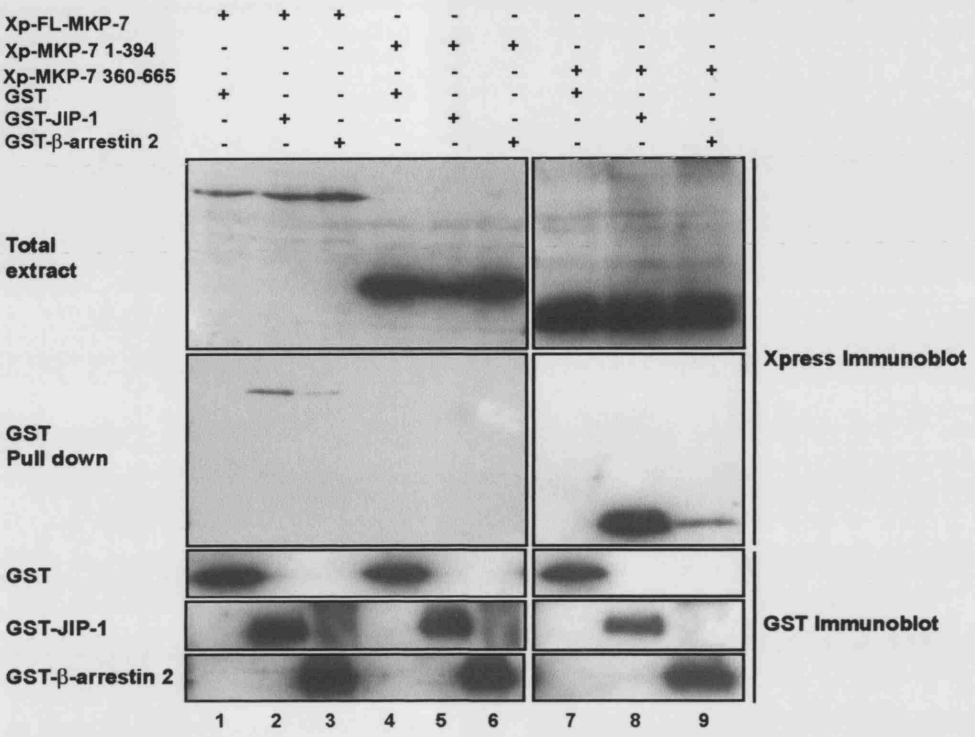

Figure 25 – 4.2.2 MKP-7 C terminal fragment is sufficient for binding both JIP-1 and beta-arrestin 2

Plasmids containing GST, GST-JIP-1 or GST-β-arrestin 2 (0.2μg) were expressed in 293T cells together with Xp-tagged MKP-7, MKP-7 360-665 or MKP-7 1-394 (0.75μg). Extracts were made the following day from 5 x 10^6 cells and GST precipitates were isolated using glutathione-sepharose beads (*GST Pull down*). These samples were then separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of MKP-7 in the GST precipitates was examined by immunoblot using the anti-Xpress tag antibody. All relative protein expression levels are also shown in the *total extracts*.

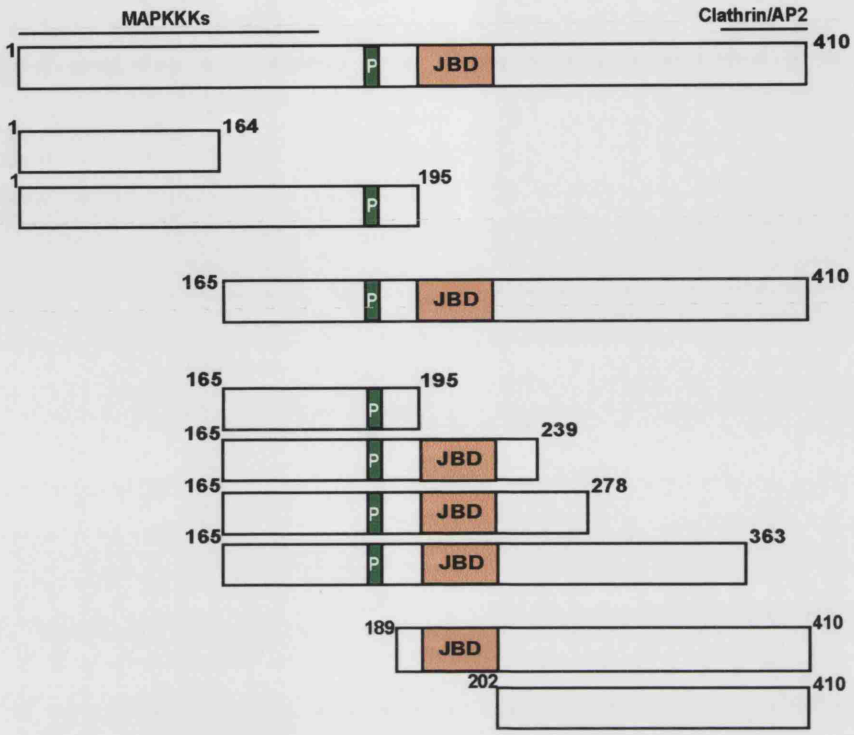

Figure 26 – 4.2.3 beta-arrestin 2 deletion constructs

Cartoon which shows β-arrestin 2 deletion constructs made and used to analyse the binding site on β-arrestin 2 for MKP-7. *Numbers* refer to the amino acid position. *JBD*, proposed JNK binding domain; *P*, phosphate sensor; regions that bind MAPKKKs and clathrin/AP2 are also indicated.

MKP-7 bound weakly to residues 1-164 and full length β-arrestin 2 (fig. 27, lanes 2 and 4), however removal of this region of β-arrestin 2 greatly enhances MKP-7 binding (fig. 28, lanes 2 and 3). These data identify a strong interaction between MKP-7 and a region between amino acids 165 and 410 of β-arrestin 2; this interaction may be regulated by binding of MKP-7 to the N terminal region of full-length β-arrestin 2. To map the strong interaction site, further β-arrestin 2 constructs were analysed. Residues between 195 and 202, previously identified as the JNK binding domain (Miller et al., 2001) were critical for strong binding (fig. 29, lanes 2 and 4, fig. 30, lanes 4 and 6). To assess the role of JNK in this interaction, we examined the binding between the β-arrestin 2 central fragment and MKP-7 residues 360-665 which do not bind JNK3. Amino acids 165-239 of β-arrestin 2 bound the MKP-7 360-665 (C terminus) (fig. 31, lane 5); this demonstrates an interaction between the MKP-7 C terminus and the central JNK binding domain region of β-arrestin 2 that is independent of JNK3. As suggested above, removal of the N terminal region (1-164) of β-arrestin 2 increases binding to MKP-7. It is possible that the N terminal region of β-arrestin 2 may regulate MKP-7 binding in the intact protein; for example a conformational change in β-arrestin 2 that displaces the N terminal region might further expose the strong binding region between amino acids 195 and 202.

4.2.4 MKP-7 specifically dephosphorylates JNK associated with β-arrestin 2

β-arrestin 2 binds JNK3 and its upstream kinases, ASK1 and indirectly MKK4, and enhances JNK3 phosphorylation (McDonald et al., 2000; Miller et al., 2001). I

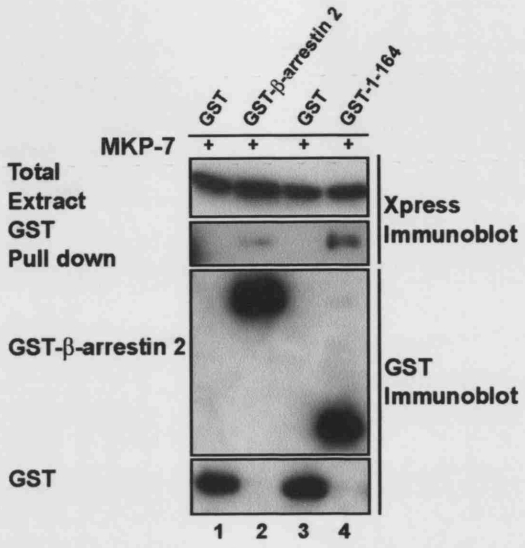

Figure 27 – 4.2.3 MKP-7 binds residues 1-164 of beta-arrestin 2

Constructs expressing GST, GST-β-arrestin 2 and GST-β-arrestin 2 deletion constructs shown in figure 26 (1-164) (0.2μg) were expressed together with Xp-tagged MKP-7 (0.75μg) in 293T cells. The following day extracts were made from 5 x 10^6 cells and β-arrestin 2-containing complexes were isolated using glutathione-sepharose beads (*GST Pull down*). The protein samples were separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane the precipitates were analysed for the presence of MKP-7 by immunoblot using the anti-Xpress tag antibody. Relative protein expression levels are also shown in the *total extracts*.

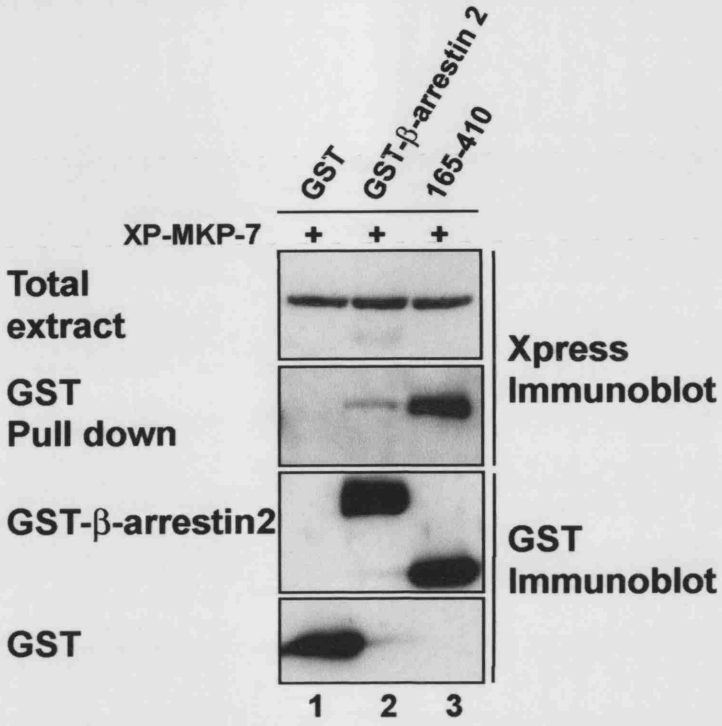

Figure 28 – 4.2.3 MKP-7 binds strongly to residues 165-410 of beta-arrestin 2

Plasmids expressing GST, GST-β-arrestin 2 or GST-β-arrestin 2 deletion construct 165-410 (shown in figure 26) (0.2μg) were expressed together with Xp-tagged MKP-7 (0.75μg) in 293T cells. The following day extracts were made from 5 x 10^6 cells and GST-containing precipitates were obtained using glutathione-sepharose beads (*GST Pull down*). These were then separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of MKP-7 was examined by western blot using the anti-Xpress tag antibody. The presence of all expressed proteins is also shown in the *total extracts*.

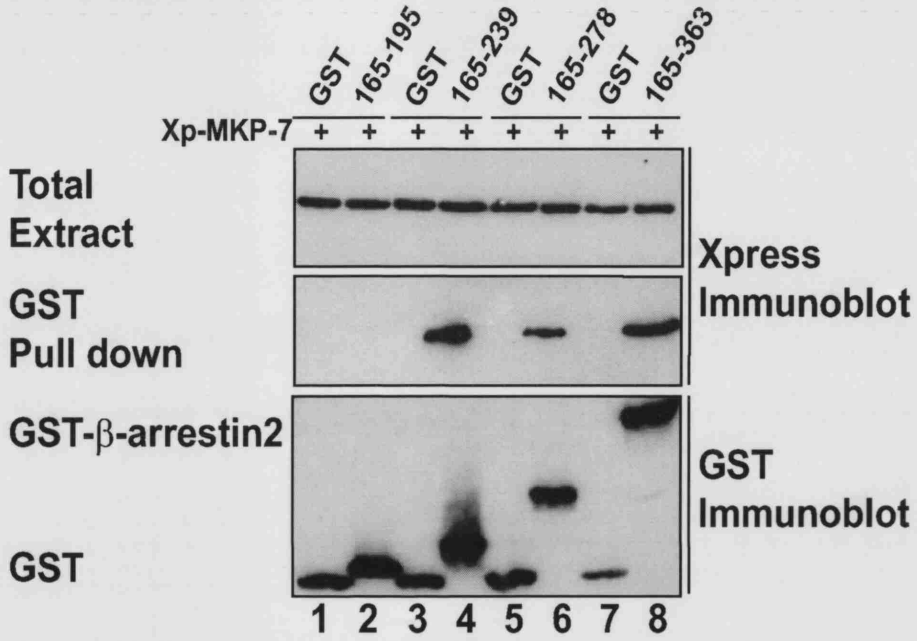

Figure 29 – 4.2.3 Residues 165-195 of beta-arrestin 2 are not required for binding MKP-7

Constructs expressing GST, GST-β-arrestin 2 or GST-β-arrestin 2 deletion constructs 165-195 to 165-363 (shown in figure 26) (0.2μg) were expressed together with Xp-tagged MKP-7 (0.75μg) in 293T cells. The following day extracts were made from 5 x 10^6 cells and β-arrestin 2-containing precipitates were obtained using glutathione-sepharose beads (*GST Pull down*). These were then separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of MKP-7 was examined by immunoblot using the anti-Xpress tag antibody. The relative expression of all the proteins used is also shown in the *total extracts*.

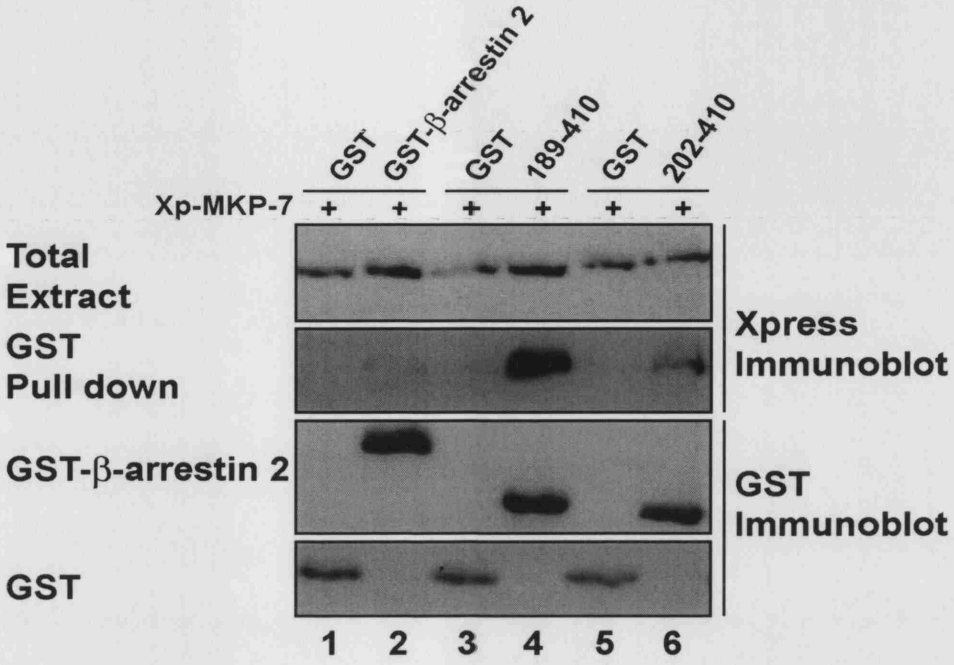

Figure 30 – 4.2.3 Residues 189-202 of beta-arrestins 2 are important for the interaction with MKP-7

Constructs containing GST, GST-β-arrestin 2 and GST-β-arrestin 2 deletion constructs 189-410 and 202-410 (shown in figure 26) (0.75μg) were expressed together with Xp-tagged MKP-7 (0.75μg) in 293T cells. The next day extracts were made from 5 x 10^6 cells and β-arrestin 2-containing precipitates were isolated using glutathione-sepharose beads (*GST Pull down*). These samples were then separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane the presence of MKP-7 was examined by immunoblot using the anti-Xpress tag antibody. The relative expression levels of all the constructs are also shown in the *total extracts*.

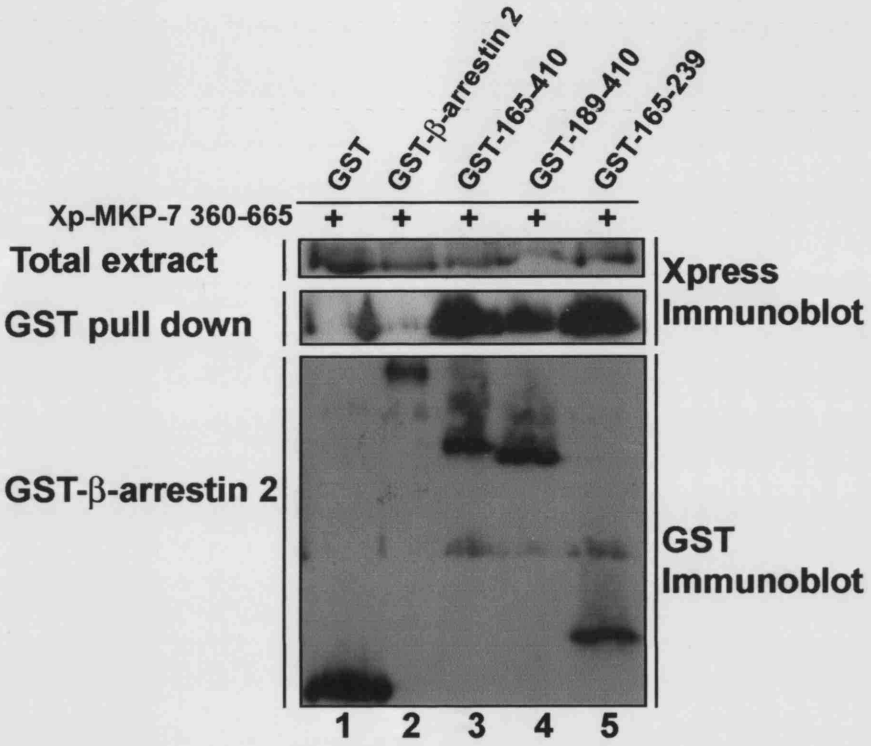

Figure 31 – 4.2.3 Binding between MKP-7 residues 360-665 and beta-arrestin 2 deletion constructs demonstrates an interaction independent of JNK

Plasmids expressing GST, GST-β-arrestin 2 and GST deletion constructs 165-410, 189-410 and 165-239 (shown in figure 26) (0.2μg) were expressed along with Xp-tagged MKP-7 residues 360-665 (0.75μg) in 293T cells. The next day extracts were made and GST-containing precipitates were isolated using glutathione-sepharose beads (*GST Pull down*). The precipitates were separated by SDS-PAGE and after electrotransfer to nitrocellulose membrane examined for the presence of MKP-7 by immunoblot using the anti-Xpress tag antibody. All protein expression levels are also shown in the *total extracts*.

therefore assessed whether MKP-7 could dephosphorylate β-arrestin 2 bound JNK, as previously described for JIP-1 bound JNK (Willoughby et al., 2003). Through over-expressing JNK3 with β-arrestin 2 and the MAP3K ASK1 in COS-7 cells, I observed enhancement of JNK activation as reported (McDonald et al., 2000) (fig. 32, lanes 2 and 3). The addition of MKP-7 inhibited the JNK3 activation enhanced by β-arrestin 2, whereas MKP-7 did not affect phosphorylated JNK in the absence of the scaffold (fig. 32, lanes 2 & 4 and 3 & 5). This activity of MKP-7 was dependent on its phosphatase activity, as a catalytically inactive mutant (Willoughby et al., 2003) did not affect JNK3 activation (fig. 33, lanes 3 and 5). These data show that MKP-7 can specifically dephosphorylate the pool of JNK3 bound to β-arrestin 2.

Angiotensin II binding to the AT1aR stimulates JNK3 phosphorylation in the presence of β-arrestin 2 (McDonald et al., 2000). I co-expressed JNK3, β-arrestin 2 and AT1aR in COS-7 cells and showed that angiotensin II stimulated phosphorylation of JNK3 occurs at 15 and 30 minutes, with the signal disappearing after 60 minutes (fig. 34, lanes 3 and 4), similar to previously published data (McDonald et al., 2000). In the absence of β-arrestin 2 and addition of MKP-7, stimulation of JNK3 phosphorylation remains the same as above (fig. 34, lanes 8 and 9). However, when MKP-7 was expressed along with β-arrestin 2, JNK3 phosphorylation was equivalent at 15 minutes after angiotensin II stimulation, but almost completely abrogated 30 minutes after stimulation (fig. 34, lanes 3, 4 and 13, 14). These data indicate MKP-7 utilises the interaction with β-arrestin 2 to

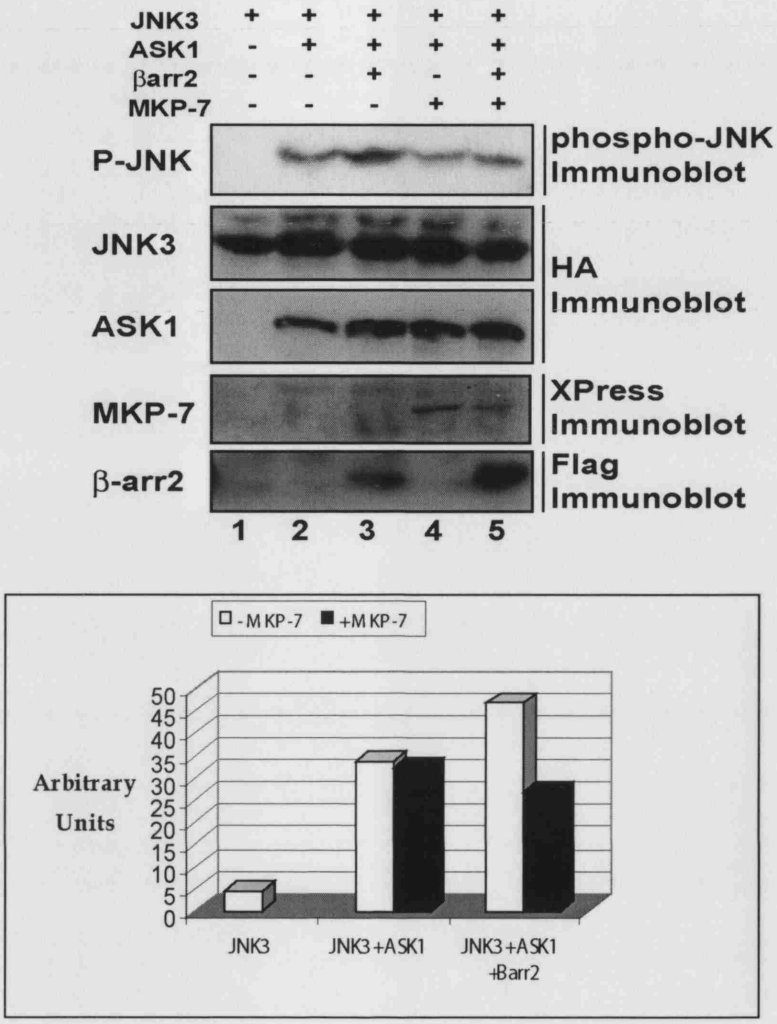

Figure 32 – 4.2.4 MKP-7 specifically dephosphorylates JNK associated with beta-arrestin 2

Constructs containing HA-tagged JNK3 (0.1μg), HA-tagged ASK1 (0.1μg), Flag-tagged β-arrestin 2 (0.2μg) and Xp-tagged MKP-7 (0.1μg) were expressed in COS-7 cells overnight. 5 x 10^7 COS-7 cells were then lysed directly into gel loading buffer and, following separation by SDS-PAGE electrotransfer to nitrocellulose membrane, were examined for the presence of the phosphorylated form of JNK3 by immunoblot using an anti-phospho JNK antibody. Relative protein expression levels are also shown in the *total extract*. The graph represents the level of JNK3 phosphorylation under each condition as arbitrary units assigned by a densitometer.

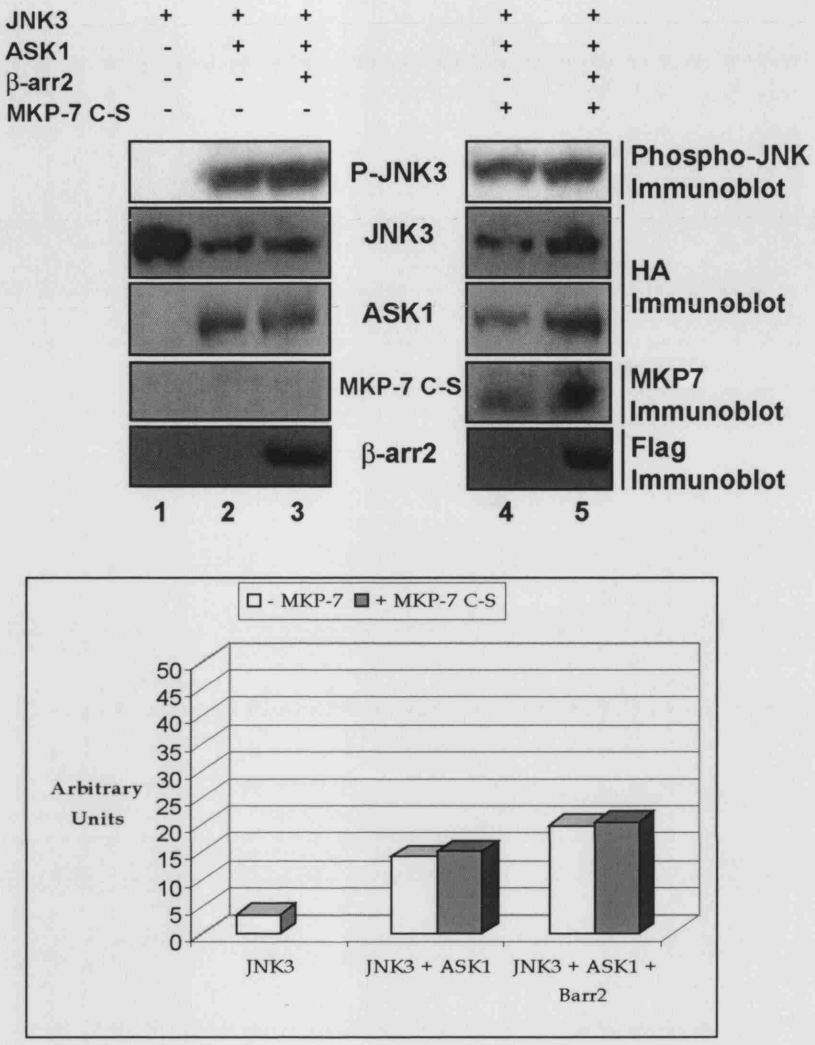

Figure 33 – 4.2.4 Catalytically inactive MKP-7 is unable to dephosphorylate beta-arrestin 2 associated JNK

Plasmids expressing HA-tagged JNK3 (0.1μg), HA-tagged ASK1 (0.1μg), Flag-β-arrestin 2 (0.2μg) and Xp-MKP-7 C-S (0.1μg) were expressed in COS-7 cells. The following day 5 x 10^7 COS-7 cells were lysed directly into gel loading buffer and the proteins then separated by SDS-PAGE. After electrotransfer the samples were examined for the presence of the phosphorylated form of JNK3 by immunoblot using an anti-phospho JNK antibody. Expression levels of all proteins used is also shown in the *total extract*. The graph represents the level of JNK3 phosphorylation under each condition as arbitrary units assigned by a densitometer.

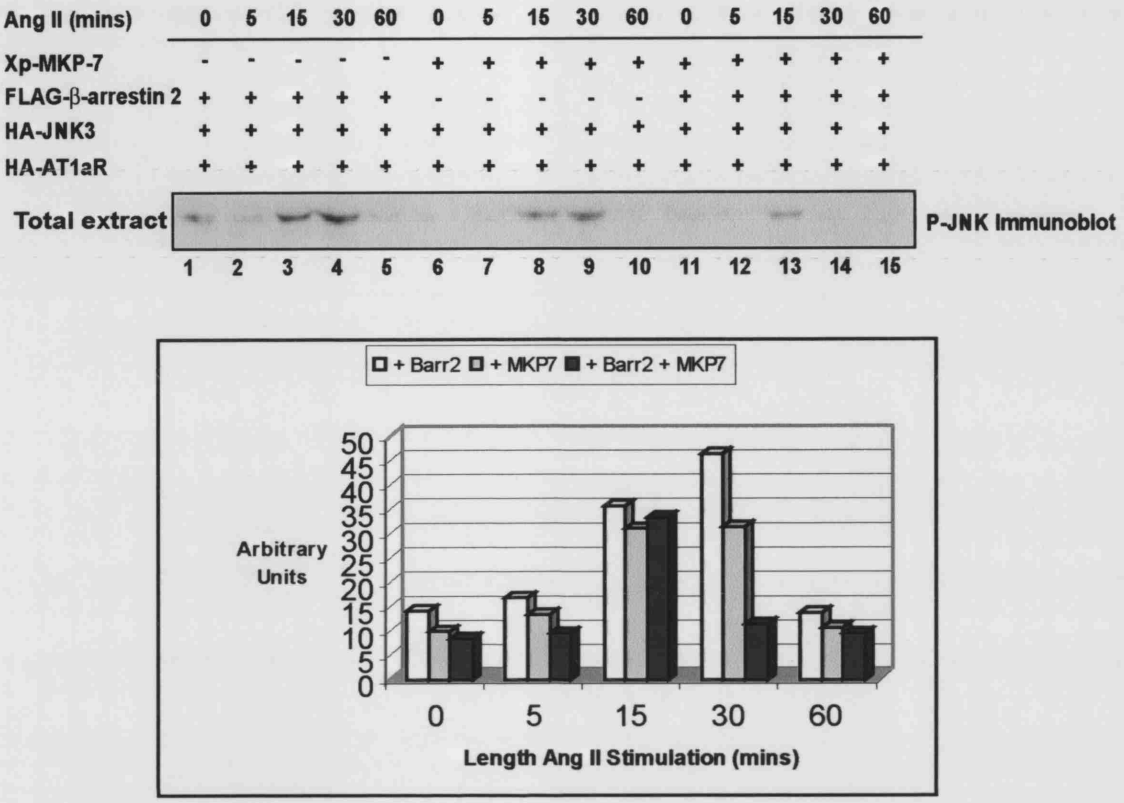

Figure 34 – 4.2.4 MKP-7 specifically dephosphorylates beta-arrestin 2 associated JNK 30 minutes after AT1aR activation

Constructs expressing HA-tagged JNK3 (0.2μg), HA-tagged AT1aR (0.4μg), Flag-tagged β-arrestin 2 (0.3μg) and Xp-tagged MKP-7 (0.1μg) were expressed in COS-7 cells. After 48 hours the cells were stimulated for the indicated time periods with 1μM Angiotensin II. 5 x 10^7 cells were then directly lysed into gel loading buffer and the proteins separated by SDS-PAGE. After electrotransfer to nitrocellulose membrane, the presence of the phosphorylated form of JNK was examined by immunoblot using an anti-phospho JNK antibody. Relative expression levels of all proteins were also examined (data not shown). The graph represents the level of JNK3 phosphorylation under each condition as arbitrary units assigned by a densitometer.

specifically dephosphorylate JNK3; thus allowing a more transient JNK3 phosphorylation under AT1aR activation.

4.2.5 MKP-7 interacts with β-arrestin 2 in resting cells but is released after AT1aR stimulation

The initial equivalent JNK3 activation seen in the presence of MKP-7, followed by its rapid inactivation, might be explained by the hypothesis that the MKP-7 interaction with β-arrestin 2 is regulated by AT1aR stimulation. To detect MKP-7 location I used a previously described polyclonal antiserum (Willoughby et al., 2003), that detects a cytoplasmic protein in transfected COS-7 cells (fig. 36). Using leptomycin B to inhibit the nuclear export of MKP-7 (Masuda et al., 2001; Tanoue et al., 2001), I demonstrated the resulting nuclear fluorescence accumulation to confirm the specificity of this staining (fig. 35). I then co-expressed MKP-7 with components of the AT1aR signaling system and β-arrestin 2. Figure 36 shows over expressed GFP-β-arrestin 2 (*green*) and MKP-7 (*red*) were both present in the cytoplasm of resting cells. Angiotensin II binding to AT1aR has been reported to cause internalisation of JNK3 bound to β-arrestin 2 on endocytic vesicles (McDonald et al., 2000). Figure 36 shows that β-arrestin 2 (*green*) moved to the plasma membrane 5 minutes after agonist activation of the AT1aR while MKP-7 remained cytoplasmic. After 15 minutes green vesicles containing β-arrestin 2 but not MKP-7 had formed, but after 30 minutes the vesicles appeared yellow indicating the recruitment of MKP-7. After 60 minutes, yellow endosomal structures had begun clustering at the perinuclear region of the cell. These

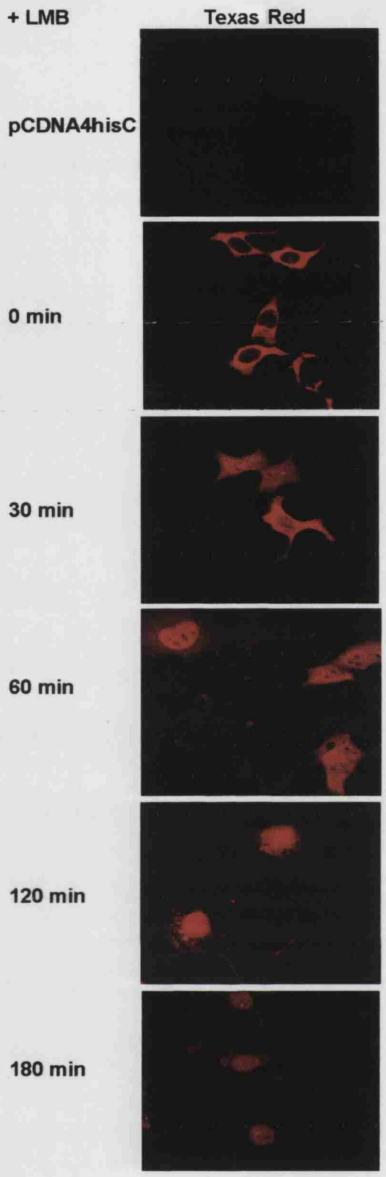

Figure 35 – 4.2.5 Leptomycin B staining confirms specificity of MKP-7 polyclonal antibody

293T cells were transfected with either pCDNA4hisC (control) (0.5μg) or untagged MKP-7 (0.5μg). The next day cells were serum starved overnight and the following day incubated with 40nM Leptomycin B for the indicated time periods. Cells were then washed with phosphate-buffered saline (PBS) solution, fixed with 4% paraformaldehyde and lysed with 0.2% Triton X-100/PBS. MKP-7 was detected using an anti-MKP-7 polyclonal antiserum with a Texas Red conjugated donkey anti-rat secondary antibody. Immunofluorescence shows MKP-7 (*red*).

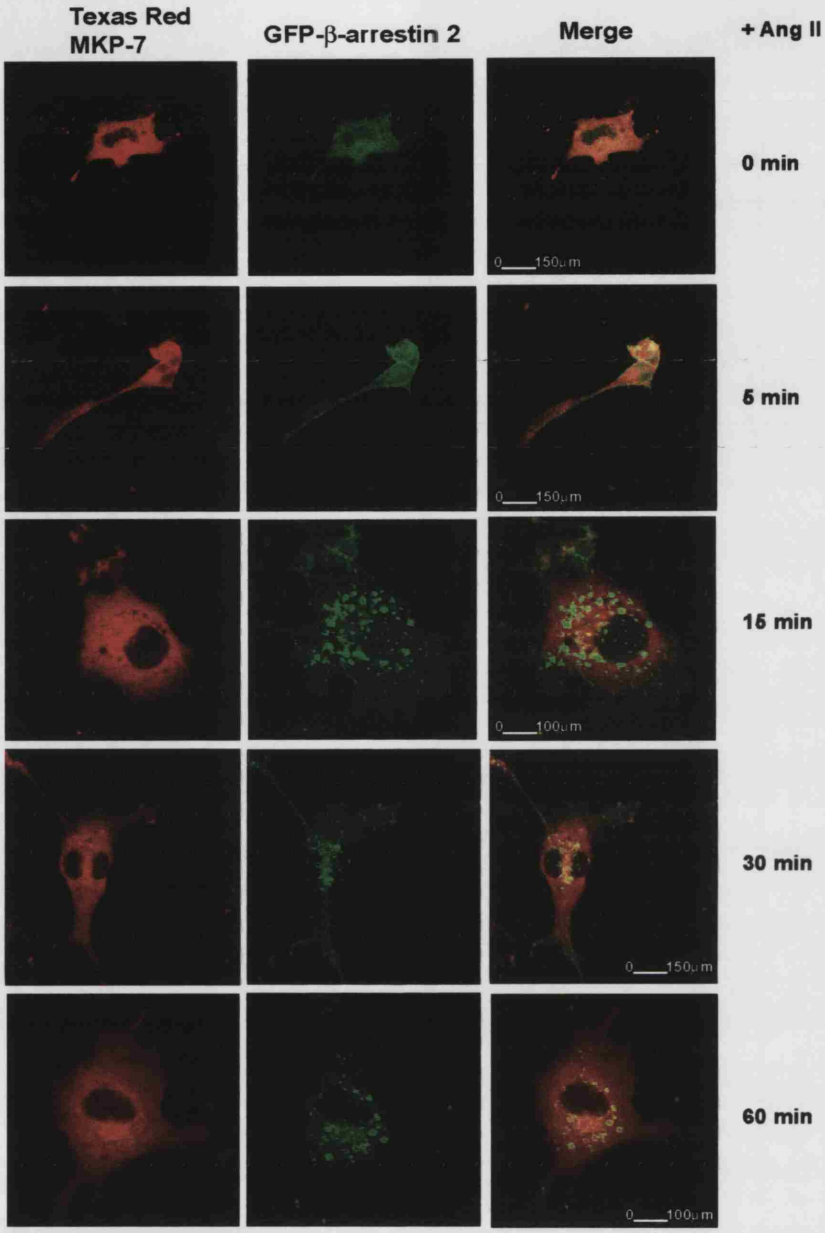

Figure 36 – 4.2.5 MKP-7 interacts with beta-arrestin 2 in resting cells, but after AT1aR activation MKP-7 is rapidly released and then re-associates with beta-arrestin 2 after 30-60 minutes

Constructs expressing GFP-β-arrestin 2 (0.4μg), untagged MKP-7 (0.4μg), HA-tagged JNK3 (0.4μg) and HA-tagged AT1aR (0.4μg) were expressed in COS-7 cells. After 48 hours, cells were stimulated for the indicated time periods with 1μM Angiotensin II. Cells were then washed with phosphate-buffered saline (PBS) solution, fixed with 4% paraformaldehyde and lysed with 0.2% Triton X-100/PBS. MKP-7 was detected using an anti-MKP-7 polyclonal antiserum with a Texas Red conjugated donkey anti-rat secondary antibody. Double-label immunofluorescence shows GFP-β-arrestin 2 (*green*) and MKP-7 (*red*) and a merged image (*yellow*).

experiments suggest that MKP-7 is released from β-arrestin 2 after activation of the AT1aR and is then re-recruited after approximately 30 minutes to β-arrestin 2 present on endocytic vesicles.

In addition, to confirm this dynamic interaction between β-arrestin 2 and MKP-7, I examined MKP-7 binding to β-arrestin 2 after AT1aR stimulation. For these experiments I used 293T cells to express sufficient protein for detection after GST pull down, rather than the COS-7 cells in which we measured subcellular localisation and JNK3 phosphorylation. In the absence of JNK3, the binding of MKP-7 to β-arrestin 2 changed relatively little after angiotensin II stimulation (fig. 37, lanes 3-7). However with the addition of JNK3, MKP-7 binding to β-arrestin 2 was lost rapidly after angiotensin II stimulation, returning to the initial level between 30-60 minutes (fig. 37, lanes 9-13) (data not shown). The level of JNK3 binding to β-arrestin 2 remains the same throughout the time course as previously described (McDonald et al., 2000) (data not shown). The presence of JNK does not effect the level of MKP-7 binding to β-arrestin 2 (fig. 37, compare lanes 2 and 8). Together, these data demonstrate that activation of the AT1aR causes rapid dissociation of MKP-7 from β-arrestin 2. This dissociation is dependent on the presence of JNK3 and is reversed 30-60 minutes after AT1aR stimulation.

As JNK3 is required for efficient MKP-7 dissociation from β-arrestin 2 triggered by angiotensin II, it is possible JNK3 activation is sufficient for MKP-7 dissociation. To do this, I expressed JNK3 along with the MAP3K ASK1 and as figure 38 shows (compare lanes 2 and 4, & 3 and 5) the presence of ASK1 causes a significant decrease in the amount of MKP-7 bound to β-arrestin 2, suggesting that

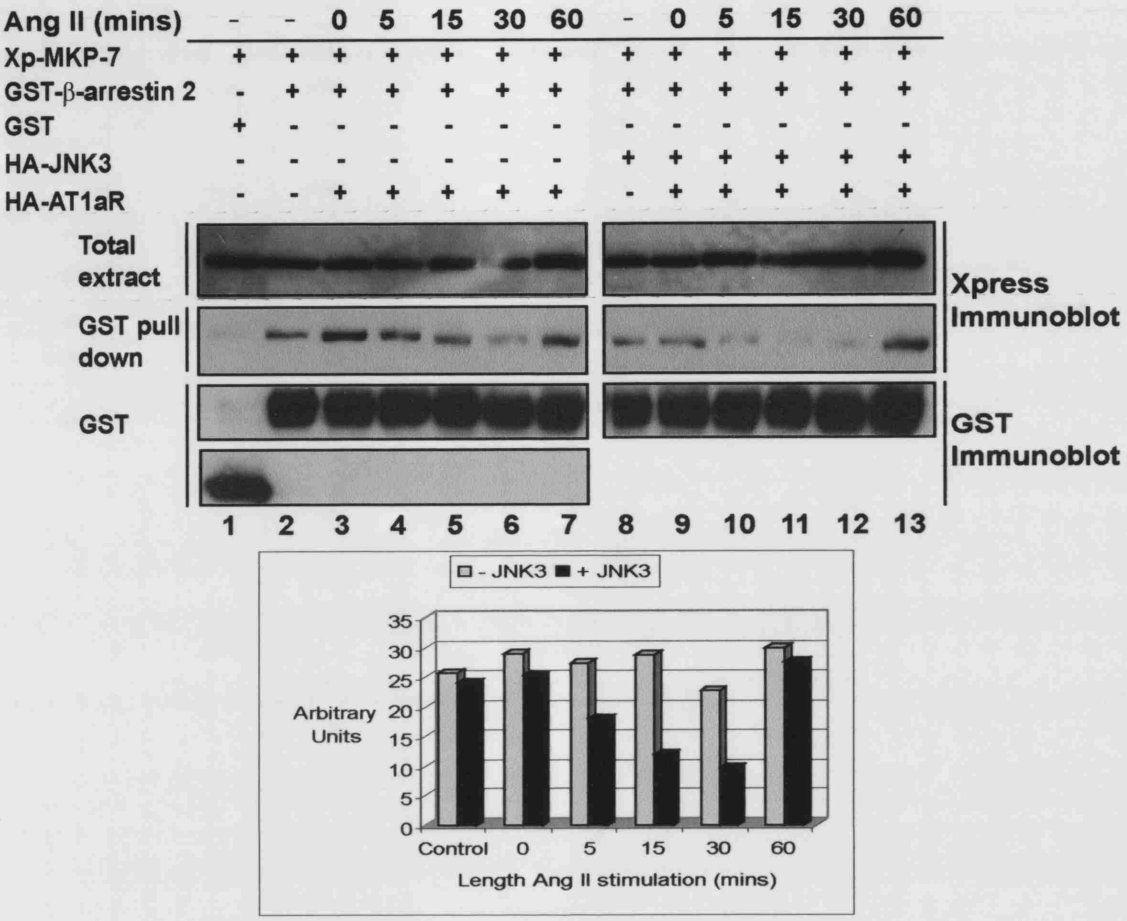

Figure 37 – 4.2.5 The interaction between MKP-7 and beta-arrestin 2 is dynamic

Plasmids containing GST (0.2μg), GST-β-arrestin 2 (0.2μg), HA-tagged JNK3 (0.75μg), HA-tagged AT1aR (0.4μg) and Xp-tagged MKP-7 (0.75μg) were expressed in 293T cells. The following day cells were stimulated with 1μM Angiotensin II for the indicated time periods and extracts made from 5 x 10^6 cells. GST-containing precipitates were isolated using glutathione-sepharose beads (*GST Pull down*) and separated using SDS-PAGE. After electrotransfer to nitrocellulose membrane, the precipitates were examined for the presence of MKP-7 and JNK3 (data not shown) by immunoblot using the anti-Xpress tag antibody. Relative expression levels are shown in the *total extract*. The graph represents the level of binding between MKP-7 and beta-arrestin 2 at each time point as arbitrary units assigned by a densitometer.

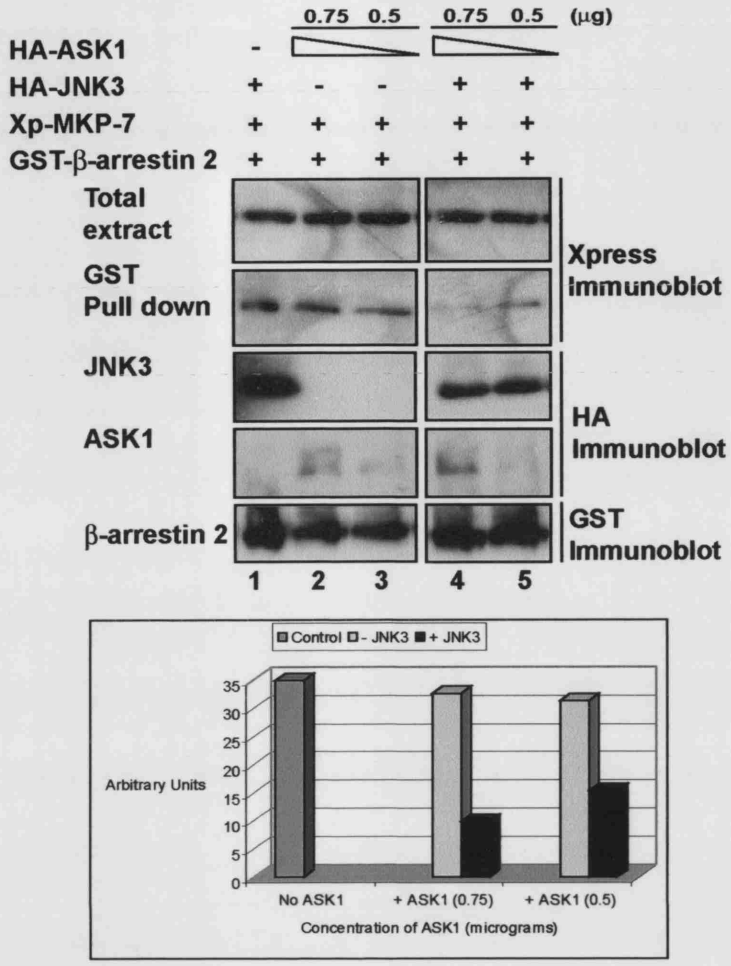

Figure 38 – 4.2.5 The presence of the MAP3K ASK1 also leads to dissociation of MKP-7 from the beta-arrestin 2

GST-beta-arrestin 2 (0.2μg) was expressed along with Xp-tagged MKP-7 (0.75μg) and HA-tagged JNK3 (0.75μg) and/or HA-ASK1 (0.75-0.5μg) in 293T cells. The next day GST containing complexes were isolated using glutathione-sepharose beads (*GST Pull down*) and separated using SDS-PAGE. After electrotransfer to nitrocellulose membrane, the presence of MKP-7 was examined by immunoblot using the anti-Xpress tag antibody. Relative expression levels of all plasmids used are also shown. The graph represents the level of MKP-7 binding to beta-arrestin 2 in the presence of different concentrations of ASK1 calculated by densitometry and showing levels as arbitrary units.

it is the stimulation of JNK3 phosphorylation that leads to MKP-7 dissociation from β-arrestin 2. The presence of ASK1 alone does not effect the interaction between MKP-7 and β-arrestin 2 (fig. 38, compare lanes 1 and 2).

4.3 Discussion

Work in this chapter has identified an interaction between DSP MKP-7 and JNK3 scaffold β-arrestin 2, through residues 394-443 of MKP-7 previously identified as the JIP binding domain (chapter 3) and a central portion of β-arrestin 2. This is a specific interaction as M3/6, which also binds JIP-1 and JIP-2, was not detected in the β-arrestin 2 precipitates. The interaction of this region of MKP-7 with both of these diverse scaffold proteins suggests that residues within it may function as a general scaffold-binding domain. This will be discussed in chapter 5. This interaction has been shown to be dynamic, as binding is lost after AT1aR activation and subsequent recruitment of β-arrestin 2 to the plasma membrane, as well as through over expression of ASK1. This transient interaction also leads to the specific dephosphorylation of JNK3 activated by over expression of ASK1 and AT1aR stimulation. After 30-60 minutes, depending on the cell system, MKP-7 re-associates with β-arrestin 2 to specifically dephosphorylate JNK present on endocytic vesicles.

Recent data has indicated that β-arrestin 2, upon activation by binding to the phosphorylated C term of GPCRs, undergoes a conformational change (Xiao et al., 2004). They suggest this conformational change results in increased β-arrestin 2 affinity for its substrate clathrin. The presence of JNK3 on the scaffold is necessary for efficient MKP-7 dissociation. Furthermore, in the presence of ASK1 MKP-7 dissociates from β-arrestin 2 suggesting JNK3 phosphorylation is required for this effect. Therefore, this conformational change in β-arrestin 2 could also be involved in the triggering of JNK3 activation and indirectly lead to the dissociation of MKP-

7 from the complex. The work here suggests a mechanism of how a signal can be transmitted through a scaffold protein that apparently binds both activating and inhibitory components.

These studies have been achieved using over expressed signaling components, however an endogenous interaction between β-arrestin 2 and JNK3 has been seen in brain lysates (McDonald et al., 2000), where MKP-7 is also highly expressed (Masuda et al., 2001; Tanoue et al., 2001). It is important an endogenous interaction between β-arrestin 2 and MKP-7 is detected to confirm the true significance of these results.

Numerous receptors have been found which utilise β-arrestin 2-type scaffold proteins to co-ordinate both the kinases and phosphatases required for receptor function. These include the β-adrenergic receptors (Fraser et al., 2000; Shih et al., 1999), AT1R (Ali et al., 2000) and mGluRs (Brakeman et al., 1997). Data in chapter 3 suggests the JNK scaffold JIP3 can bind members of the DSP family, most significantly MKP-3. JIP3 is proposed to play a role in LPS activation of JNK through TLR4 which indicates the potential role for JNK regulation by DSPs in another receptor signaling system. This regulation of scaffold bound molecules by DSPs could therefore be a common theme. Furthermore, 7TM receptors Fizzled (Wilbanks et al., 2004) and Smoothened (Chen et al., 2004), as well as non-GPCRs such as TGFβ (Chen et al., 2003), are coupled to β-arrestin 2 scaffold proteins. It is therefore possible that MKP-7 and other DSPs can regulate MAPK signaling in a number of receptor signaling systems.

Chapter 5: Discussion and future directions

5.1 General

The work presented in this thesis describes the novel interactions between the JNK scaffold proteins, JIP and β-arrestin 2, and specific members of the dual specificity phosphatase family. These scaffold proteins have been shown to interact with JNK MAPK and specific upstream signaling components (McDonald et al., 2000; Whitmarsh et al., 1998). When over expressed, the formation of these complexes has been shown to enhance JNK activity which can subsequently lead to enhanced c-Jun activation. In some of the cases described here, as well as in published work (Willoughby et al., 2003), the interaction between JNK scaffold and DSP leads to the specific dephosphorylation of scaffold-bound JNK which also results in the reduction of c-Jun phosphorylation. In particular, these data identify the DSP, MKP-7, as being able to co-operate with both JIP-1 and β-arrestin 2 to negatively regulate JNK. Furthermore, data presented here suggests MKP-7 can transiently regulate GPCR activated JNK through a dynamic interaction with β-arrestin 2 (Willoughby and Collins, 2005). These data hereby suggest a mechanism by which a positive signal can pass through a JNK scaffold complex which binds both positive and negative regulators.

5.2 Residues required for scaffold/phosphatase interaction

Work performed in chapters 3 and 4 identify a group of residues in MKP-7 as being required for binding JIP-1 and β-arrestin 2. Specifically, a site between 394 and 443, in the C terminal extension of MKP-7 contain residues required for binding to JIP-1 and β-arrestin 2. Areas within this region are highly conserved through mouse and human MKP-7 as shown in figure 39 (a), particularly towards the amino end of this region. Interestingly, as expected, the two sequences show high homology throughout. This region contains the DSP catalytic domain (also shown in fig. 39 (a)) and CDC25 regions. These domains are characteristic of DSPs, highly conserved through family members and different species and critical to MKP function. The homology between mouse and human MKP-7 394-443 suggests residues within this site are important to MKP-7 function and therefore have been conserved. They could in fact represent a potential scaffold binding domain. Further determination of different species sequences for MKP-7 will be required to fully analyse this apparent significance.

MKP-7 is one of a pair of closely related DSPs, the other being M3/6/VH5, which contain an extended C terminal region. Data presented here and in published work (Willoughby et al., 2003) shows that these two phosphatases are the only DSP members able to bind to JIP-1 & -2. Although not presented in this thesis, it has been identified that M3/6 also binds to JIP-1 through residues within its C terminal domain. The alignment in figure 39 (b) shows the homology between MKP-7 and M3/6. In addition, MKP-7 interacts with β-arrestin 2 through the same region as with JIP-1, whereas M3/6 is not detected. This might be due to the

Figure 39 (a) – 5.2 A clustalW alignment of MKP-7 mouse and human amino acid sequences

An alignment of the amino acid sequences of mouse and human MKP-7. The region 394-443 (underlined) shows areas of high homology between the two species at the amino acid level as shown; it also controls MKP-7 binding to the JNK scaffolds JIP-1 and beta-arrestin 2. The catalytic motif is also underlined (HCXXXXXR). *Numbers* refer to amino acid position; * indicates that the amino acids are identical; : indicate conserved amino acid substitutions; . indicate semi-conserved amino acid substitutions; spaces indicate no conservation.

Figure 39 (b) – 5.2 A clustalW alignment of human MKP-7 and M3/6 (VH5) amino acid sequences

An alignment of the amino acid sequences of human MKP-7 and VH5. The region important in MKP-7 for binding scaffold proteins (394-443) is underlined and part of it shows high homology to a region in the C terminal extension of VH5. The catalytic motif is also underlined (HCXXXXXR). *Numbers* refer to amino acid position; * indicates that the amino acids are identical; : indicate conserved amino acid substitutions; . indicate semi-conserved amino acid substitutions; spaces indicate no conservation.

fact that MKP-7 may contain unique residues which specify binding to β-arrestin 2 which M3/6 does not contain. In addition, the level of M3/6 binding to JIP-1 is lower than that of MKP-7 (data not shown) and furthermore, MKP-7 binds to β-arrestin 2 more weakly than it binds to JIP-1 (fig. 21, *lanes 2 and 4*). Therefore, the level of detection may not be sufficiently strong enough to detect the presence of M3/6 in the β-arrestin 2 precipitates if binding is proportionally equal. Figure 39(b) shows an amino acid sequence alignment of human MKP-7 and human M3/6 - VH5. This alignment shows that even though these two DSPs are closely related, there is sequence divergence. Along with the DSP catalytic domain (also shown in fig. 39(b)), another homologous sequence exists within the alignment as a small number of amino acids contained within the 394-443 region (amino acids 396-406). Analysis of the amino acid sequences of M3/6 and VH5 indicate these regions are also highly homologous (data not shown). Two potential ideas therefore exist. It is possible this small region contains amino acids critical for both MKP-7 and M3/6 binding to JIP-1 and -2, and β-arrestin 2 (although not detected with M3/6), which are conserved through this type of DSP. However, it may be the residues missing from VH5 within the 394-443 region but present in MKP-7 which control binding to β-arrestin 2. Furthermore, the alignments between both mouse and human MKP-7, and MKP-7 and VH5 show that residues present before 394-443 are also homologous. It may therefore be the disruption of these and 394-443 site which leads to loss of binding to scaffold and they therefore may also play a role in scaffold binding. I have attempted to analyse this complete region for any well characterised binding motifs but have been unsuccessful. Interestingly, in this

study I identified MKP-3 as being able to bind the JIP-3 isoform. I examined the amino acid sequence of MKP-3 and performed a sequence alignment together with MKP-7 and found very little homology between the two proteins or any residues in MKP-3 resembling residues in the 394-443 region of MKP-7. I have also performed alignments between the portions of JIP-1 (282-471) and β-arrestin 2 (central domain) responsible for binding these phosphatases. However, these did not identify any significant sites which may be responsible for binding the phosphatases. Time permitting, I would like to gain the skills to carry out a more detailed bioinformatic search to further my understanding of how these interactions occur and whether it is through a common site. This would include further biochemical analysis of each residue to pin point the exact residues required for binding in both the scaffolds and the phosphatases.

As described above, the binding of MKP-7 to JIP-1 and β-arrestin 2 works through a region within the C term of the phosphatase. The binding site within MKP-7 for JNK lies in its amino terminus and this interaction works through distinct docking domains present on both proteins (Tanoue et al., 2002). This indicates MKP7 does not bind to the scaffold via an interaction with JNK. Analysis of JIP-1 confirms this interaction is independent of JNK as removal of the JNK binding domain barely affects MKP-7 binding to JIP-1 (Willoughby et al., 2003). Further analysis of JIP-1 identifies a site independent to that of JNK binding on JIP-1 which controls binding of MKP-7. Although in β-arrestin 2 the JNK3 binding site has been elucidated, the site of ASK1 binding remains as a general region within the N terminal region of β-arrestin 2 (Miller et al., 2001). Data presented here

suggests MKP-7 also binds to β-arrestin 2 independently of JNK. Further work to identify specific residues on both JIP-1 and β-arrestin 2 present for binding MKP-7 is required. This work may uncover specific phosphatase binding domains which would be crucial to our understanding of how all these proteins, including the kinases, interact in relation to one another.

5.3 Proposed model of the dynamic interaction between β-arrestin 2 and MKP-7

As presented in chapter 4, a transient interaction exists between the β-arrestin 2 scaffold protein and DSP MKP-7. The degree of this interaction depends on the activation state of JNK, as through AT1aR stimulation and ASK1 over expression the interaction between these proteins is lost (figs. 37 and 38, respectively). Although it is unclear how this occurs, it is possible a conformational change in β-arrestin 2, resulting from its binding to the C tail of AT1aR, allows the phosphorylation of JNK which causes the release of MKP-7 from the scaffold complex as represented in figure 40. This dissociation permits a period of scaffold-bound JNK activation. This mechanism could explain how the transient stimulation of JNK3 phosphorylation can be seen in the presence of MKP-7.

The cumulative result of figures 34, 36 and 37 suggests a time course for this dynamic interaction; from loss of MKP-7 under AT1aR stimulation at 0-5 minutes to an average time of 30 minutes where MKP-7 re-associates with the scaffold complex. It is important to point out, that there are variations in these results which could be attributed to the use of two different cell lines. For example, in COS-

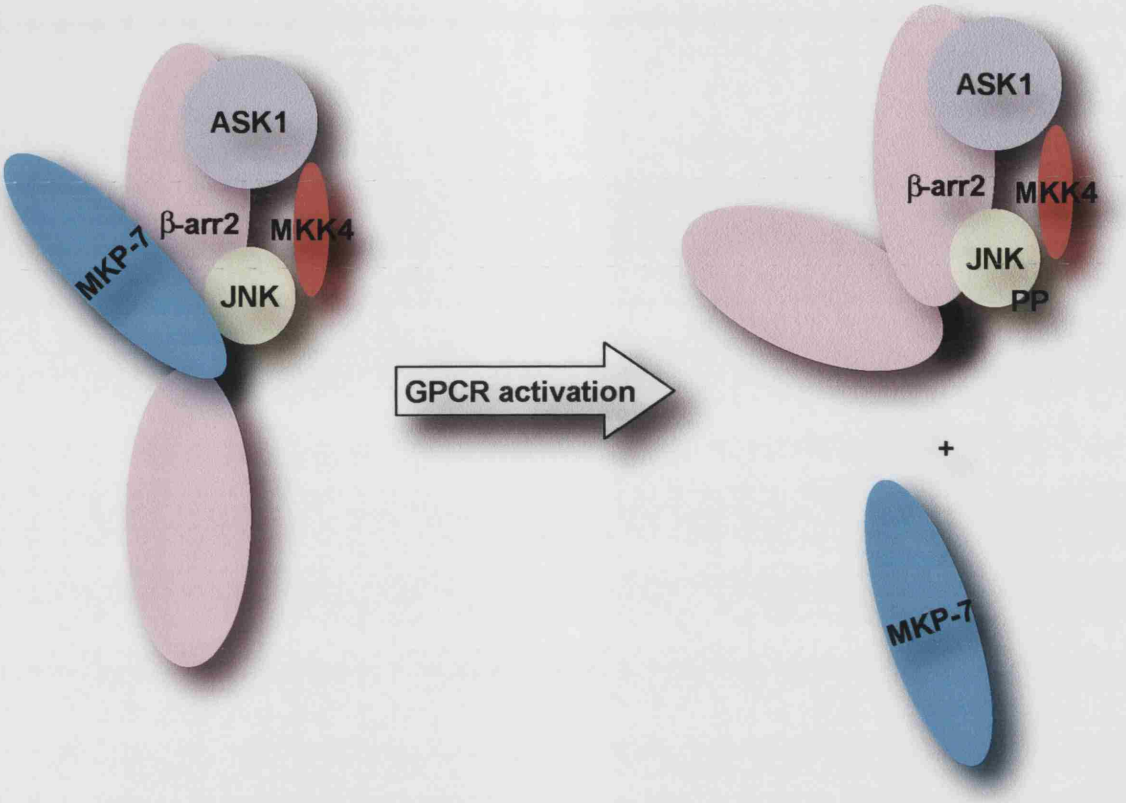

Figure 40 – 5.3 Theoretical model proposing the changes required for MKP-7 dissociation from beta-arrestin 2 under GPCR and JNK3 activation

This work hypothesises that the loss of MKP-7 from the scaffold may require the activation of JNK3. This activation may be the result of a conformational change in beta-arrestin 2, which might be stabilised by an affinity between the phosphate sensor region located in the centre of beta-arrestin 2 (●) and the phosphorylated C tail of the GPCR (not shown).

7 cells both the immunofluorescence and phosphorylation studies indicate MKP-7 re-association between 15-30 minutes, whereas in 293T this occurs between 30-60 minutes. Obviously, a comparison of these experiments performed separately in each cell line would be an advantage in determining a precise time course for the interaction and how greatly the results from the two cell lines differ.

Figure 36 shows the general subcellular distribution of the complex during AT1aR stimulation and the re-association of MKP-7 and β-arrestin 2 on endocytic vesicles occurring between 15-30 minutes. With this in mind, figure 41 illustrates a proposed model which indicates the possible locations of these events after stimulation of AT1aR. Preliminary experiments presented here identify that active JNK can contribute to the loss of MKP-7 from β-arrestin 2. Further work is required to fully understand the components and conditions required for this to occur. This includes analysis of the presence of a non-activatable JNK on the scaffold and its affect on MKP-7 binding. However, the mechanism of how MKP-7 re-associates with β-arrestin 2 on endocytic vesicles, at the time points described above, requires extensive further work. If indeed activated JNK is required for the loss of MKP-7 from the complex then it could be assumed a disruption to JNK could lead to the re-association of MKP-7. This could be due to loss of ligand from GPCR through acidification of the vesicle or dephosphorylation of the C tail of the GPCR by an unknown phosphatase, resulting in diminished ASK1, and therefore JNK, activation. This is highly speculative and even if true it is very likely specific components and conditions are also required. Future studies are crucial in determining how MKP-7 re-associates with β-arrestin 2.

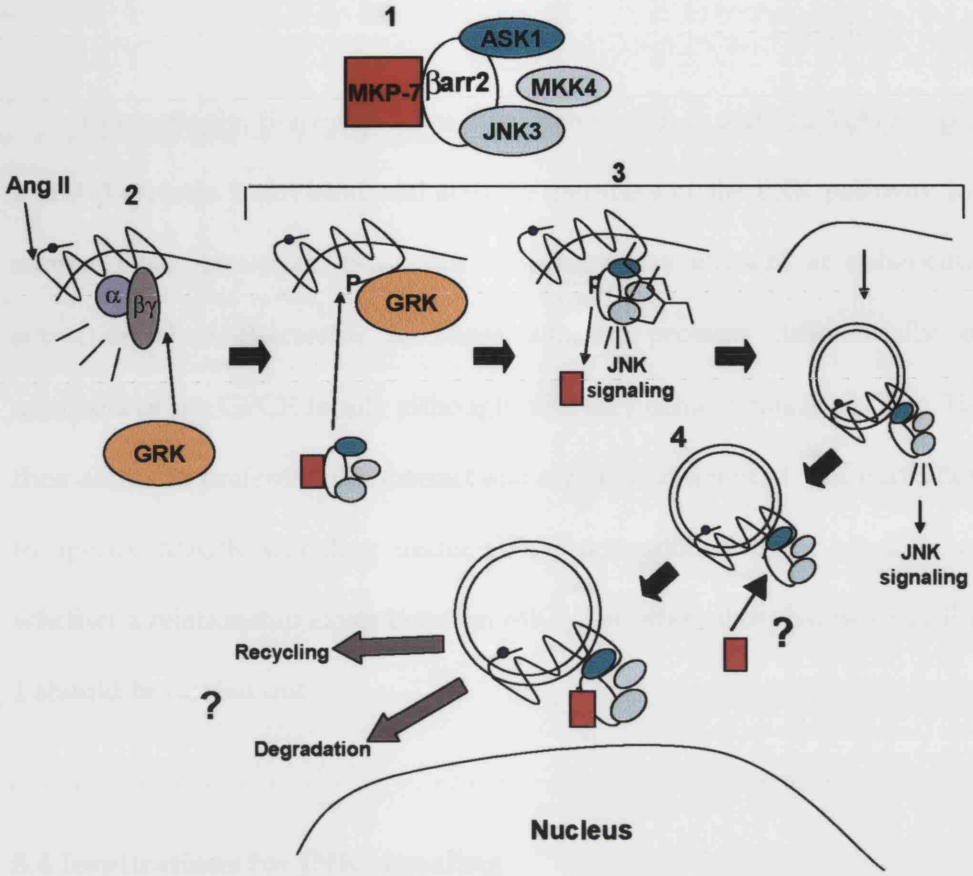

Figure 41 – 5.3 Theoretical model representing the location of events between MKP-7 and beta-arrestin 2 under GPCR ligation

1, in a resting cell β-arrestin 2 binds MKP-7. **2**, upon agonist stimulation the AT1aR recruits G proteins which in turn recruit G protein-coupled receptor kinases (GRKs) which phosphorylate Ser/Thr residues within the C terminal tail. **3**, this phosphorylation recruits β-arrestin 2 to the plasma membrane along with JNK3, MKK4 and ASK1, to associate with the activated AT1aR, where it physically blocks any further G protein signaling (desensitisation). JNK3 bound to β-arrestin 2 is activated and begins signaling, whereupon MKP-7 binding to β-arrestin 2 is lost. **4**, AT1aR bound β-arrestin2 and the JNK3 cascade are trafficked onto endosomal vesicles. MKP-7 is recruited back to the scaffold in order to regulate JNK3 potentially through diminished ASK1 and therefore JNK activation. Depending on the environment AT1aR is either slowly trafficked back to the plasma membrane or destroyed through lysosomal degradation.

? – indicates the next step/mechanism is unclear.

Interestingly, β-arrestin 1 has also been shown to scaffold MAPKs. β-arrestin 2 and β-arrestin 1 can bind and activate members of the ERK pathway to a very similar level. However, β-arrestin 1 appears less efficient at enhancing JNK3 activation than β-arrestin 2. These adaptor proteins differentially regulate members of the GPCR family although how they achieve this is unclear. Therefore their ability to preferentially interact and enhance different MAPK may allow them to specify MAPK signaling under GPCR activation. Further work to examine whether a relationship exists between MKP-7 or other phosphatases and β-arrestin 1 should be carried out.

5.4 Implications for JNK signaling

5.4.1 JNK and scaffold proteins

As described in chapter 1, activated JNK is known to regulate its substrates upon translocation to the nucleus. The classical JNK response exists through its activation of the transcription factor c-Jun which is able to regulate gene expression and trigger responses including apoptosis. The interaction of JNK with numerous scaffold proteins suggests JNK regulation can be tightly controlled. Most JNK scaffold proteins localise, due to distinct binding properties (the kinesin light chain in the case of the JIP proteins), to specific subcellular compartments as described in chapter 1. They accumulate the correct MAPK components and insulate them from other signaling modules. This allows them to control the environment surrounding the JNK module and therefore the outcome of JNK

substrate activation. Evidence suggests JNK activity is enhanced through binding to these scaffold complexes when compared to non-scaffold bound JNK. Once released from the scaffold complex this enhanced active JNK is allowed to enter the nucleus where it targets its substrates. However, scaffold proteins only bind to a proportion of the total population of JNK present in a cell. Therefore, the reason for binding scaffolds may be a mechanism whereby under certain conditions a JNK module can step up a gear and relay a rapid signal. A single JNK signaling module can utilise multiple scaffold proteins and in this way potentially respond rapidly to many different signals. Ultimately, analysis of the physiological roles of JNK scaffold proteins, especially the JIPs, will help us to understand the relationship between these complexes and JNK signaling. In particular, confirmation of the components of endogenous JNK scaffolds under physiologically relevant conditions, including MKP-7, needs to be achieved. It is also necessary to determine the full structure of these scaffolds in order to understand spatial distributions of each component. This will help us to understand the mechanism of MAPK activation through the use of scaffold proteins.

As suggested above, scaffold proteins localise JNK modules to distinct subcellular locations and in this way maintain JNK in a non-nuclear location. It is unclear whether whilst bound to a scaffold protein activated JNK can target cytosolic substrates. Identification of cytosolic substrates, if any, of scaffold-bound JNK is very important and would reveal further insight in to the function of these scaffold complexes. In particular, during GPCR stimulation does JNK3 associated

with β-arrestin 2 target cytosolic substrates? In the case of ERK, G protein signaling via ERK occurring during GPCR stimulation is responsible for activation of transcription factor EGR-1, whereas β-arrestin 2-bound ERK activation is retained in the cytosol suggesting targeting to non-nuclear substrates (Pearson et al., 2001; Wei et al., 2004). It is therefore important to assess whether activation of c-Jun and other nuclear substrates occurs or is enhanced in the presence of β-arrestin 2 bound JNK3.

5.4.2 MKP-7

Evidence including the data presented here shows specific JNK scaffolds, as well as binding positive regulators, are able to interact with negative factors to influence JNK activation. Protein phosphatases can regulate activated MAPK in the nuclear compartment. However, the DSP MKP-7, which binds both JIP-1 and β-arrestin 2, has the ability to shuttle in and out of the nucleus. As scaffold proteins retain active JNK in the cytosol for a period of time, the data here suggests JNK may also require negative regulation in the cytosol as well as the nucleus. This cytosolic regulation therefore again implies JNK may have cytosolic substrates. As identified in chapter 1, these substrates could include MAPs, 14-3-3 proteins and Bcl-2 family member BAD.

This relationship between MKP-7 and different JNK scaffolds identifies that there are some members of the DSP family that can in fact regulate their substrates outside of the nucleus. Compared to other members of the DSP family, including MKP-1 and MKP-3, MKP-7 is not rapidly induced by any tested stimuli. This

suggests MKP-7 protein exists at a constant low level. This idea fits with the notion that MKP-7 is easily degraded through the proteosome and that therefore separate modifications are required to help stabilise the cellular level of this protein (Katagiri et al., 2005). It is therefore possible that under the correct stimuli, stabilisation of the protein occurs which allows MKP-7 protein accumulation. This increase in MKP-7 concentration can result in the formation of a MKP-7 scaffold-complex and/or its translocation to the nucleus where in potentially both situations it can regulate JNK. The ability of MKP-7 to regulate JNK3 associated β-arrestin 2 after only 15 minutes also provides evidence for its maintenance at low levels within the cell and that its activity is not dependent on its transcriptional regulation. However, only analysis of MKP-7 at the physiological level will determine whether this is true or not.

It has also been suggested that MKP-7 links a feedback pathway between ERK and JNK. As described in chapter 1, work has shown that active ERK can phosphorylate MKP-7 within its C terminal region which stabilises this protein by preventing degradation. The presence of MKP-7, whether bound to JNK scaffold proteins or not, will lead to the inactivation of JNK. This ability of activated ERK therefore prevents activation of JNK and allows the ERK pathway to remain dominant. This relationship could be a mechanism of how the duration and result of a response are controlled.

It is clear different cell types utilise MAPK signaling pathways differently, therefore over expressed systems can only suggest possible physiological situations. Here using over expression studies has allowed the examination of

fundamental properties of MKP-7 interactions with JNK scaffolds. However, the lack of effective reagents able to detect endogenous MKP-7 severely inhibits our ability to study the physiological function of this phosphatase. Future useful studies would include the production of an MKP-7 null mouse and a high affinity anti-MKP-7 antibody for use at the endogenous level. Our lab was unable to produce a highly efficient anti-MKP-7 antibody as shown in figure 12. SiRNA, to knockdown MKP-7 protein levels, would also prove a useful technique although identifying a cell with particular interest for MKP-7 and sufficient detection tools would be required first.

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